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Rapid Genotyping of Hepatitis C Virus by Primer-Specific Extension Analysis

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Quick and accurate genotyping of hepatitis C virus (HCV) is becoming increasingly important for clinical management of chronic infection and as an epidemiological marker. Furthermore, the incidence of HCV infection with mixed genotypes has clinical significance that is not addressed by most genotyping methods. We have developed a fluorescence-based genotyping assay called primer-specific extension analysis (PSEA) for the most prevalent HCV genotypes and have demonstrated the capacity of PSEA-HCV for detecting mixed-genotype HCV infections. PSEA-HCV detects genotype-specific sequence differences in the 5′ untranslated region of HCV in products amplified by the COBAS AMPLICOR HCV Test, v2.0. Simulated mixed HCV infection of plasma with RNase-resistant RNA controls demonstrates that PSEA-HCV can detect as many as five genotypes in one specimen. Furthermore, in dual-genotype simulations, PSEA-HCV can unequivocally detect both genotypes, with one genotype representing only 3.1% of the mixture (313/10,000 IU in starting plasma). Compared to INNO-LiPA HCV II, both assays determined the same genotype for 191/199 (96%) patient specimens (175 subtype and 16 genotype-only identifications). Following the initial evaluation, PSEA-HCV was used routinely to genotype HCV from patient specimens submitted to our laboratory (*n* = 312). Seventeen (5.4%) mixed infections were identified. The distribution of single-infection HCV genotypes in our population was 60.9% type 1 (*n* = 190), 12.8% type 2 (*n* = 40), 20.2% type 3 (*n* = 63), 0.3% type 4 (*n* = 1), and 0.3% other (*n* = 1). In conclusion, PSEA-HCV provides an inexpensive, high-throughput screening tool for rapid genotyping of HCV while reliably identifying mixed HCV infections.

Infection by hepatitis C virus (HCV) is the leading cause of chronic liver disease worldwide (19). The overall prevalence of HCV infection in the United States is 1.8%, with most of the patients unaware of their infection and at risk for developing cirrhosis and hepatocellular carcinoma (15, 21). HCV is a positive-sense, single-stranded RNA virus that displays extensive genetic heterogeneity (1). At least six major HCV genotypes comprising numerous, more closely related subtypes have been identified (26). HCV genotypes display significant differences in their global distribution and prevalence, making genotyping a useful method for determining the source of HCV transmission in an infected localized population (11). Furthermore, in addition to viral load and liver histology, the genotype of the infecting HCV strain appears to be an important determinant of the severity and aggressiveness of liver infection, as well as patient response to antiviral therapy (26). Consequently, several methods for genotyping HCV have been developed, including direct DNA sequencing (2, 5, 6), type-specific PCR (17), restriction fragment length polymorphism (16), line probe assays (22, 23), primer-specific and mispair extension analysis (7, 8), heteroduplex mobility analysis by temperature gradient capillary electrophoresis (14), and denaturing high-performance liquid chromatography (13).

The majority of the HCV genotyping assays in use today were designed to identify only the dominant HCV genotype in a sample and consequently are unable or limited in their ability to identify multiple genotypes in patients infected with more than one strain of HCV. Hu et al. (7) have found that of the HCV infections determined to contain mixed genotypes by DNA sequencing, only 36%, 17%, and 14% were accurately identified as mixtures by type-specific PCR, line probe, and restriction fragment length polymorphism analyses, respectively. Furthermore, DNA sequencing itself was unable to detect 40% of the mixed-genotype infections and could not reliably detect genotypes that were present in mixtures at levels below 25% (7). Consequently, most currently available genotyping methods are unable to assess the prevalence of mixed-genotype infections, which may have a significant impact on the interpretation of clinical studies addressing genotype-specific responses to interferon and interferon combination therapies. As injection drug use is the major risk factor for HCV infection in Canada, accounting for 71% of the cases (3), the occurrence of mixed-genotype infections may be more common than previously reported, as this route of transmission may result in multiple exposures to different HCV strains in habitual users. Moreover, individuals infected with HCV via repeated blood transfusions are also potential carriers of multiple HCV genotypes.

We have developed a novel genotyping method to identify the most prevalent genotypes among HCV-infected Canadians, which is based on primer-specific extension analysis (PSEA) of HCV PCR amplicons, called PSEA-HCV. That is, primers specific for genotypes 1, 2, 3, and 4 and subtypes 1a, 1b, 2a/c, and 2b were designed to bind to variable regions within the amplified 5′ untranslated region (UTR) of the HCV genome. The PSEA-HCV assay, utilizing the inefficiency of *Taq* DNA polymerase to

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TABLE 1. Primers for HCV genotyping by PSEA-HCV

Identifier	Sequence[a]	Amt (ng)/reaction	Product length[b] (bp)
PSEA-HCV-1	5′ HEX- AAG GAC CCG GTC GTC CT 3′	6	128
PSEA-HCV-2	5′ FAM- TAT CCA AGA AAG GAC CCA 3′	15	137
PSEA-HCV-3	5′ FAM- CAA CAC TAC TCG GCT AGT 3′	2	200
PSEA-HCV-4	5′ HEX- CAT GGC GTT AGT ATG AGT GTT 3′	60	229
PSEA-HCV-1a	5′ HEX- ACT CGG CTA GCA GTC TT 3′	5	193
PSEA-HCV-1b	5′ FAM- ACT CGG CTA GCA GTC TC 3′	0.5	193
PSEA-HCV-2a/c	5′ HEX- GAG TAC ACC IGA ATT GCC GGG 3′	1.25	151
PSEA-HCV-2b	5′ FAM- TGA GTA CAC CGG AAT TMC CG 3′	5	152
PSEA-HCV-IC	5′ FAM- CCG GTT CCG CAG ACC ACT 3′	1.6	93

[a] 5′ fluorescent labels: HEX, 6-carboxy-2′, 4, 4′, 5′, 7, 7′-hexachlorofluorescein; FAM, 6-carboxyfluorescein.
[b] Based on extension reactions using the 244-bp product defined by primers KY78 and KY80 (12).

extend a mismatch at the 3′ end of a primer, has demonstrated predicted products against known HCV genotypes. Another primer targeted for a conserved region among all genotypes was designed for use as an internal control (IC). We report here on the PSEA strategy and its performance against known HCV genotypes typed by INNO-LiPA HCV II (23). Simulated mixed infections were also assayed by PSEA-HCV, and this work demonstrates the ability of this novel method to identify multiple genotypes and in ratios of up to 31:1.

MATERIALS AND METHODS

Samples. All plasma was separated from whole blood within 6 h of collection, aliquoted, and stored at −70°C until testing or shipment on dry ice. A total of 511 HCV-positive plasma samples were genotyped by PSEA and were from Saskatchewan patients specifically queued for HCV genotyping between 1998 and 2004. Plasma from the first consecutive 199 samples were also sent to the National Microbiology Laboratory of Canada for HCV genotype confirmation by INNO-LiPA HCV II (Innogenetics, Ghent, Belgium, now distributed by Bayer Diagnostics NAD as the VERSANT HCV Genotype Assay).

Qualitative RT-PCR. The COBAS AMPLICOR HCV Test, v2.0 (Roche Diagnostics, Branchburg, NJ), was used according to the manufacturer's instructions to detect HCV RNA. Essentially, HCV RNA was isolated from the plasma of 511 HCV-infected Saskatchewan patients by lysis of virus particles with a chaotropic agent, followed by precipitation of the RNA with alcohol. The AMPLICOR HCV Test uses reverse transcription (RT)-PCR with primers KY80 (5′-GCA GAA AGC GTC TAG CCA TGG CGT-3′) and KY78 (5′-CTC GCA AGC ACC CTA TCA GGC AGT-3′) to amplify a 244-nucleotide region of the 5′ UTR of the HCV genome. The otherwise discarded A-ring, containing the resulting 244-bp amplicon in positive samples, was saved for PSEA.

Simulated mixed-genotype infections. Hepatitis C Virus Panel I (Ambion Diagnostics, Austin, TX) provided the RNase-resistant RNA controls for HCV genotypes 1a, 1b, 2a/c, 2b, and 3a that were used singly and in different proportions for the simulations. The COBAS AMPLICOR HCV MONITOR Test, v2.0 (Roche Diagnostics, Branchburg, NJ), was used according to the manufacturer's instructions to quantify the levels of HCV RNA in each control. A multiple mixed-genotype infection was simulated by mixing equal proportions of each RNA control. Two different dual-genotype infections containing either 3a/1b or 1a/1b were simulated by mixing the following proportions of either genotype 3a or 1a with genotype 1b: 0:100 (100%), 1:1 (50%), 1:3 (25%), 1:7 (12.5%), 1:15 (6.3%), 1:31 (3.1%), 1:63 (1.6%), and 1:127 (0.8%). All simulations were mixed such that the total copy number always equaled 2,000 IU/ml. Each mixture was then treated the same as patient plasma, therefore extracted and amplified by the COBAS AMPLICOR HCV Test before PSEA.

PSEA-HCV. (i) Primer design. Primers specific for genotypes 1, 2, 3, and 4 and subtypes 1a, 1b, 2a/c, and 2b were designed to bind to variable regions R1 to R7 (23) within the amplified 5′ UTR of the HCV genome. An additional primer was designed to bind to a highly conserved region within the amplified region of the 5′ UTR for extension on all HCV genotypes, serving as an IC for the assay. Each PSEA-HCV primer was 5′ fluorescently labeled to facilitate detection (Sigma-Genosys, Woodlands, TX). The primer sequences, the dye labels, and the sizes of the expected products are listed in Table 1. The primers are named for the genotypes they specify.

(ii) Template preparation. The amplicon-containing solution in the A-ring from the COBAS AMPLICOR HCV Test was prepared for PSEA-HCV with a Microcon YM-100 Centrifugal Filter Unit (Millipore Corporation, Billerica, MA). Briefly, the solution was passed through the filter at 2,000 × *g* for 4 min. Impurities and inhibitors were removed by adding 500 μl of diethyl pyrocarbonate-treated water to the filter and centrifuging the mixture at 2,000 × *g* for another 18 min. The filter was then inverted, and 50 μl of diethyl pyrocarbonate-treated water was added before centrifuging the inverted filter in a fresh tube at 3,000 × *g* for 6 min. The collected filtrate was typically diluted 1/10 before extension but used undiluted when peak heights in PSEA-HCV were weak.

(iii) Extension reaction. The primer-specific extension reactions for HCV genotypes 1, 2, and 3 were multiplexed with the IC primer. All other primers were run separately with the IC only. For each sample, the multiplexed reaction (i.e., 1, 2, 3, and IC) and the duplex reaction with PSEA-HCV-4 (i.e., 4 and IC) were performed and considered round 1 of the assay. Results from round 1 dictated which subtyping duplex reactions (i.e., round 2 of the assay) were run on a sample-by-sample basis. For example, specimens indicating HCV genotype 1 after round 1 would only be queued for the two separate duplex reactions with PSEA-HCV-1a and PSEA-HCV-1b.

Each 25-μl reaction mixture contained 5 μl of template, 2.5 μl of 10× reaction buffer (Applied Biosystems [AB], Foster City, CA), 1.5 or 2.5 mM $MgCl_2$ (AB), 200 μM each deoxynucleoside triphosphate (Amersham Biosciences, Amersham, United Kingdom), PSEA-HCV primers, and 1.25 U of Ampli*Taq* DNA polymerase (AB). The optimal amounts of the PSEA-HCV primers are listed in Table 1. $MgCl_2$ was used at a final concentration of 2.5 mM for the multiplexed reaction including 1, 2, 3, and the IC (i.e., reaction with PSEA-HCV-IC, PSEA-HCV-1, PSEA-HCV-2, and PSEA-HCV-3) and HCV subtyping duplex reactions 1a (i.e., reaction with PSEA-HCV-IC and PSEA-HCV-1a) and 1b (i.e., reaction with PSEA-HCV-IC and PSEA-HCV-1b). $MgCl_2$ was used at a final concentration of 1.5 mM for duplex reactions 4, 2a/c, and 2b. Extension reactions were cycled through the following thermal regimen on a 96-well GeneAmp PCR system: initial denaturation at 94°C for 20 s, followed by 25 cycles of 94°C for 20 s, 64°C for 20 s, and 72°C for 35 s.

(iv) Detection. One microliter of each primer extension product was mixed with 2 μl of a loading solution consisting of a 5:1:1 mixture of deionized formamide–25 mM EDTA with 50 mg/ml Blue Dextran–GeneScan-500 (TAMRA) size standard (AB). The mixtures were heated at 95°C for 5 min and snap-cooled, and 1.5 μl was electrophoresed on 4% polyacrylamide–6 M urea-Tris-borate-EDTA 12-cm gels for 1 h. The extension products were detected on an ABI PRISM 377 DNA Sequencer and analyzed with GeneScan 3.1 software (AB).

(v) Analysis. Electropherograms are scored for all peaks with peak height and respective color recorded in an Excel spreadsheet (available upon request). The spreadsheet standardizes all peaks to the IC peak height and automatically makes genotype calls based on the ratios. Essentially, only genotypes (i.e., peaks for 1, 2, 3, and 4) are called if the respective peak is at least 5% of the height of the IC peak. Subtyping peaks (i.e., peaks for 1a, 1b, 2a/c, and 2b) need to be at least 15% of the IC peak height. The IC peak used for each standardization is the one duplexed with the HCV-specific primer used for the analysis, and in this way any reaction-specific inhibitions or gel loading variations are compensated for.

RESULTS

PSEA is based on the detection of template-directed extension products of in vitro DNA polymerization reactions with fluorescently labeled oligonucleotides (i.e., primers). The PSEA-HCV primers have been designed to anneal to geno-

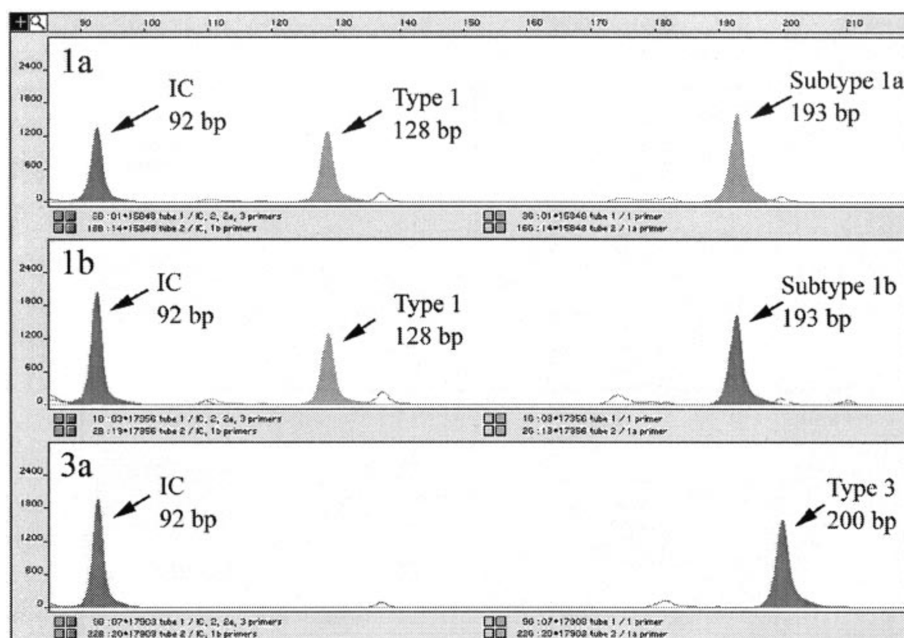

FIG. 1. Typical PSEA-HCV results. Electropherograms generated from the PSEA-HCV of HCV-positive patient plasma containing HCV genotypes 1a, 1b, and 3a. All peaks for each sample have been superimposed within the same panel to facilitate analysis. The actual peak sizes are indicated and are within 1 bp of the predicted sizes.

type-specific regions of HCV such that differences between genotypes correspond to the 3′-terminal base(s) of the primers. This novel assay capitalizes on the fact that *Taq* lacks a 3′-5′ exonuclease activity (i.e., proofreading capacity), thereby preventing the enzyme from removing a mismatched base pair at the 3′ end of a primer, which has annealed to a noncomplementary site (10). Relative to those of correctly paired bases, the extension efficiencies of terminal mismatches by *Taq* are 10^{-3} to 10^{-4} for transition and 10^{-2} to $>10^{-6}$ for transversion mispairs (9). Thus, primer extension efficiency by the *Taq* enzyme is dramatically affected by the complementation between primer and template.

The most highly conserved region of the HCV genome is the 5′ UTR, and it is often targeted so that diagnostic nucleic acid amplification tests reliably detect all genotypes (12). However, the region contains distinct genotype-specific differences in sequence, which make the 5′ UTR suitable for genotyping (24). In the PSEA-HCV assay, primers specific for genotypes 1, 2, 3, and 4 and genomic subtypes 1a, 1b, 2a/c, and 2b have demonstrated predictable binding to variable regions within the amplified 5′ UTR of the HCV genome. The PSEA-HCV assay, utilizing the inefficiency of *Taq* DNA polymerase to extend a mismatch at the 3′ end of a primer, has demonstrated predicted products against known HCV genotypes. Typical results are shown in Fig. 1.

Simulated mixed-genotype infections. The performance of PSEA, for HCV mixed-genotype detection, was assessed by simulating HCV infection with RNase-resistant viral RNA controls (25) (18). The results of PSEA-HCV on a single mixture with equal amounts of five genotypes are shown in Fig. 2. PSEA-HCV showed excellent discrimination, with extension products successfully discerning all five genotypes. Furthermore, PSEA-HCV demonstrated excellent specificity since no nonspecific products were seen in any reaction and there was no cross-reaction with the primer specific for genotype 4 on any of the other five genotypes tested.

The sensitivity of PSEA-HCV for mixed-genotype discrimination was evaluated by using dual-genotype simulations. The results of PSEA-HCV in dual-genotype analyses with genotype 1b and either 1a or 3a at doubling dilutions of the latter genotype are shown in Table 2, such that the proportion of 1a or 3a in the mixtures ranged from 50% to 0.8%. Genotype 1a was accurately detected by PSEA-HCV down to 1:15, whereas genotype 3a was unequivocally detected down to 1:31 (Fig. 3). The 1a and 1b mixed-infection simulation demonstrates that PSEA-HCV is capable of detecting a single nucleotide polymorphism with as few as 625 copies among 10,000 genome equivalents, which should approach the analytical sensitivity needed to reliably detect most mixed HCV infections.

Comparison of PSEA-HCV with INNO-LiPA HCV II. Table 3 summarizes the results from 199 consecutive Saskatchewan patients who tested HCV positive by the COBAS AMPLICOR qualitative test. The overall concordance between PSEA-HCV and INNO-LiPA HCV II was 98.5% (196/199), including 128 genotype 1, 26 genotype 2, 38 genotype 3, 2 genotype 4, and 2 concordant mixed-infection genotype determinations. There were 21 differences in genotypes when subtype information was considered, and 7 of these amounted to differences in mixed-infection calls at the subtype level. One discrepant result was due to completely different HCV genotype calls (i.e., genotype 1 instead of genotype 6). However, the other two results completely discordant between the two methods were due to mixed-infection calls by PSEA-HCV, which could have been missed by INNO-LiPA HCV II.

Prevalence of HCV genotypes in Saskatchewan, Canada. Table 4 summarizes the results from 312 consecutive

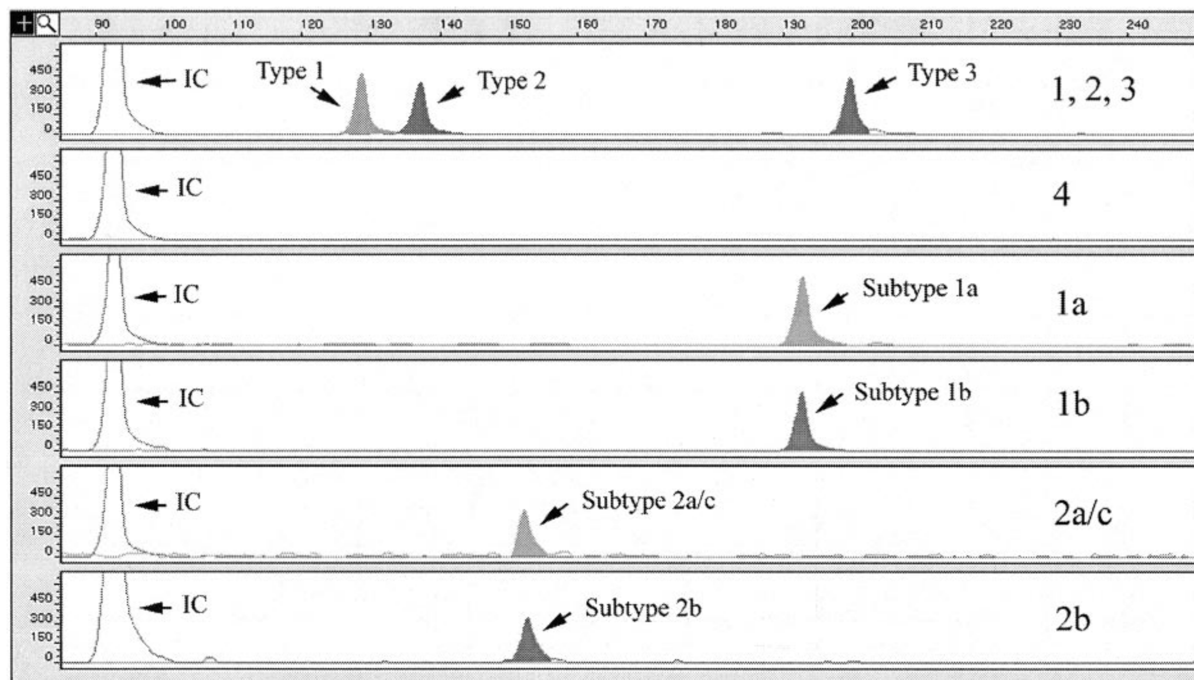

FIG. 2. Detection of multiple mixed HCV genotypes by PSEA-HCV. Five different genotypes (1a, 1b, 2a, 2b, and 3) were mixed in equal amounts and successfully detected by PSEA-HCV. The results from the six separate extension reactions are displayed as separate panels, with each panel indicating which primers were included. All panels show successful amplification with the presence of the IC. The top panel contains predicted peaks for genotypes 1, 2, and 3, whereas panel 4 shows no genotype 4 peak, as expected. The remaining panels show the expected subtyping results of genotypes 1 and 2.

Saskatchewan patients by the PSEA-HCV method. Disregarding subtyping information, the prevalences of genotypes 1, 2, 3, and 4 are 64%, 14%, 20%, and 0.3%, respectively. Three patients (0.9%) had mixed HCV types, and only one patient yielded untypeable results by PSEA-HCV. The assay successfully genotyped 99.7% (n = 311) of the HCV-positive samples but was unable to subtype 7.4% (n = 22) of either genotype 1 or genotype 2 samples as intended with the subtyping primers described. However, considering the subtyping information that was successfully obtained, an additional 14 patients tested had mixed-genotype infections at the subtype level. Consequently, a total of 17 (5.4%) patients in our population exhibited mixed-genotype infections as determined by PSEA-HCV. Therefore, on the basis of the subtyping information, the distribution of single-infection HCV genotypes in our population was 60.9% type 1 (n = 190), 12.8% type 2 (n = 40), 20.2% type 3 (n = 63), 0.3% type 4 (n = 1), and 0.3% other (n = 1). There was no statistically significant difference in the genotype distribution by age or sex in our population (data not shown).

TABLE 2. Results of PSEA-HCV on doubling dilutions of 1a or 3a with 1b in a dual-genotype simulated mixed HCV infection

Ratio of either 1a:1b or 3a:1b	Starting no. of genome equivalents[a]	% Composition of diminishing genotype	PSEA-HCV result for:	
			1a-1b	3a-1b
1:1	5,000:5,000	50.0	1a-1b	3a-1b
1:3	2,500:7,500	25.0	1a-1b	3a-1b
1:7	1,250:8,750	12.5	1a-1b	3a-1b
1:15	625:9,375	6.3	1a-1b	3a-1b
1:31	312.5:9,687.5	3.1	1b	3a-1b
1:63	156.3:9,843.8	1.6	1b	1b
1:127	78.1:9,921.9	0.8	1b	1b
NA[c]	0:10,000	0.0	1b	1b
NA	0:0	0.0	Neg[b]	Neg

[a] Copy number of armored RNA within 200 μl of spiked plasma.
[b] Neg, negative.
[c] NA, not applicable.

DISCUSSION

Sensitive and accurate detection of mixed-genotype HCV infections has become an increasingly important requirement of genotyping assays. First, the severity of hepatitis C and patient response to current antiviral therapies seem, at least in part, to be determined by the genotype of the infecting HCV strain (26). Second, mixed-genotype infections may be more common than previously reported given the typical routes of HCV infection and the inadequate sensitivity of most genotyping assays to detect them (20). Among HCV-infected Canadians, mixed genotypes have been found in 8% of HCV-positive blood donors, 14% of patients with chronic hepatitis C, and 17% of thalassemia patients who had received multiple transfusions (7). Thus, the need for HCV genotyping assays able to accurately detect mixed infections is warranted by the appreciable occurrence of such infections and their potential impact on the patient response to antiviral treatment.

PSEA-HCV was developed as a sensitive assay for the detection of the most prevalent HCV genotypes among infected North Americans. The genotype-specific primers designed for use in the PSEA-HCV assay produce predicted extension frag-

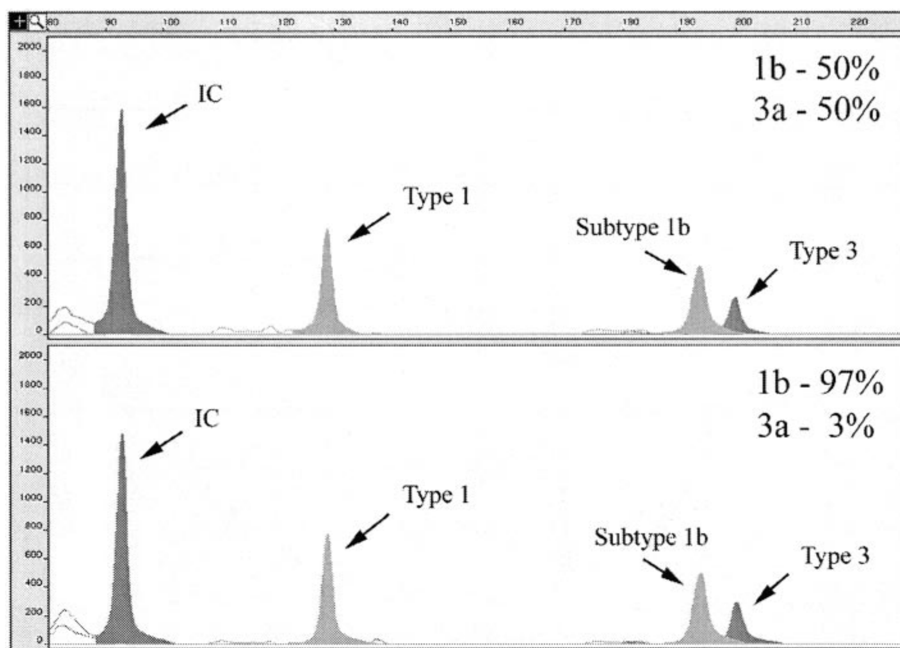

FIG. 3. Sensitivity of PSEA-HCV for mixed dual-genotype discrimination. The upper panel contains the results of PSEA-HCV on a simulated dual-genotype infection with genotypes 1b and 3a, whereby both genotypes contribute equally to the mixture totaling 10,000 genome equivalents. The lower panel contains an electropherogram that demonstrates the detection limit of PSEA-HCV for mixed-genotype detection but displays the unequivocal nature of the detected peaks.

ments based on the 5′ UTR amplified products of the COBAS AMPLICOR HCV Test, v2.0. Consequently, the PSEA-HCV assay does not require the user to re-extract the sample or repeat the amplification. This offers several advantages, including cost and time savings. Capitalizing on the workflow from a qualitative PCR test ensures an HCV genotyping sensitivity of 50 IU/ml regardless of the genotype (12) and thereby eliminates efforts to genotype HCV-negative specimens. The method is simple and rapid and can be easily performed by any laboratory equipped to perform PCR and fragment analysis on an automated DNA sequencer (e.g., ABI 377). Since PSEA-HCV is designed to multiplex the most common genotypes in one reaction, it can effectively provide HCV genotyping (i.e., 1, 2, 3, or "other") within hours of an HCV-positive result. Furthermore, the IC within the PSEA-HCV prevents "other" determinations due to reaction failure. Accordingly, PSEA-HCV has demonstrated the capacity to type 99% of the HCV genotypes seen in the Saskatchewan population in one reaction tube. The additional individual reactions required to subtype HCV genotype 1 or 2 can be set up as necessary. It is debatable whether HCV subtyping information is clinically relevant or even reliable for HCV genotypes 1a and 1b when using the 5′ UTR (4) and could be omitted from routine service work.

The comparison between PSEA-HCV and INNO-LiPA HCV II demonstrates that, with subtyping information included, there is a 96% (191/199) concordance. Disregarding subtyping information produced by both assays, the concordance increases to 99% (197/199). In either scenario, the biggest contributor to the marginal discordance between assays was mixed-infection calls. However, PSEA-HCV displays good performance in simulated mixed-genotype calls whereas the capacity for INNO-LiPA HCV II to correctly identify mixed

TABLE 3. Comparision of PSEA-HCV with INNO-LiPA HCV II

Level of concordance	Genotype(s)[a] determined by:		No. of isolates
	PSEA-HCV	LiPA	
Perfect	1a	1a	71
	1b	1b	37
	2a/c	2a/c	8
	2b	2b	12
	3	3	38
	4	4	2
	2a/c, 4	2a/c, 4	1
	1b, 3	1b, 3	1
	1a, 1b	1a, 1b	5
Total			175
Type	1	1	1
	1a	1	8
	1	1a, 1b	1
	1a, 1b	1	1
	1a	1a, 1b	1
	1a, 1b	1a	1
	1a, 1b	1b	1
	1b	1a, 1b	1
	2a/c	2	1
	2	2b	2
	2b	2	2
	2b	2a/c, 2b	1
Total			21
Discordant	1	6a	1
	1a, 4	1	1
	1b, 3	1b	1
Total			3

[a] Categorized based on permutations and combinations found and totaled.

TABLE 4. Clinical results of PSEA-HCV

Genotype(s)	No. of isolates
1	9
1a	114
1b	67
1a, 1b	10
2	11
2 a/c	13
2b	16
2a/c, 2b	4
3	63
4	1
1, 3	2
2, 4	1
ND[a]	1

[a] ND, not determined.

genotypes is poor (7). As shown here, PSEA-HCV can accurately detect the component genotypes in simulated mixed infections for samples containing as little as 3.1% of one genotype relative to the other and up to five different genotypes simultaneously. Consequently, it appears that PSEA-HCV offers a cost-effective, rapid, and sensitive method for HCV genotyping of both single and mixed infections.

The distribution of the major single-infection HCV genotypes in Saskatchewan was 60.9% type 1 (190/312), 12.8% type 2 (40/312), 20.2% type 3 (63/312), and 0.3% type 4 (1/312), which is comparable to the national average of 67%, 19%, 22%, and 2%, respectively, that has been reported previously (3). Notably, 5.4% (17/312) of the Saskatchewan patients with symptoms of liver disease tested had mixed-genotype infections, which is significantly lower than the 7.9% of HCV-infected blood donors reported previously (7). Interestingly, the prevalence of HCV genotype 4 in our population doubles with the inclusion of mixed-genotype infections. It has been shown here that mixed infections with HCV genotype 4 could be underreported by the INNO-LiPA HCV II assay. The significance of missed mixed-infection identification could likely manifest in the skewed interpretation of HCV viral load when different genotypes respond differently to antiviral therapy. Clearly, the PSEA-HCV assay appears to be a suitable alternative to established HCV genotyping assays but offers the advantage of reliable mixed-genotype detection.

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