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Original article

Evaluation of three different hepatitis C virus typing methods for detection of mixed-genotype infections

Muhammad IDREES, Irshad UR REHMAN, Sobia MANZOOR, Haji AKBAR, Sadia BUTT, Samia AFZAL, Muhammad Zubair YOUSAF & Abrar HUSSAIN

Division of Molecular Virology and Molecular Diagnostics, National Centre of Excellence in Molecular Biology, University of the Punjab, Lahore, Pakistan

OBJECTIVE: To evaluate the clinical applicability of an eligible assay for the true prevalence of hepatitis C virus (HCV) mixed-genotype infections.

METHODS: A newly developed HCV genotyping method targeting all six major HCV genotypes and 12 subtypes, restriction fragment length polymorphism (RFLP) and a serotyping assay were utilized for the detection of HCV mixed-genotype infections using known HCV genotypes and unknown samples.

RESULTS: In a defined mix of HCV genotypes, a genotype present at levels as low as 8.3% was detected by our newly developed assay, showing a threefold increase in sensitivity over that of direct deoxyribonucleic (DNA) sequencing. A comparative study of the accuracy among the three genotyping methods was carried out on samples obtained from 50 thalassemic patients who received multiple blood transfusions. The results showed that viruses in approximately 42% of the samples from this group were determined to be infected with mixed genotypes by our newly developed method. A serotyping assay and RFLP analysis, performed with poor results, could identify only 18% and 10% of mixed-genotype infections, respectively.

CONCLUSION: The newly developed assay may be the method of choice when detection of genotypes present at low levels in mixed-genotype infections due to its higher level of sensitivity.

KEY WORDS: genotyping, hepatitis C virus, mixed genotype, prevalence.

INTRODUCTION

As an RNA virus hepatitis C virus (HCV) has a high rate of genetic mutation.[1] As a result, there is extensive HCV genetic heterogeneity in infected individuals and HCV isolates are found as either a group of isolates with very closely related genomes, referred to as quasispecies or genetically distinct groups called genotypes.[2,3] At a time more than one genotype, referred to as mixed-genotype, can be found in the circulation of some HCV-infected patients, particularly in i.v. drug abusers and individuals who have received multiple transfusions.[4] The rate of HCV mixed-genotype infections is extremely variable in the same group of patients tested by different assays.[5] It is difficult to assess the true prevalence of mixed-genotype infections by currently available assays, including direct DNA sequencing, since they are designed to identify

Correspondence to: Muhammad IDREES, Division of Molecular Virology and Molecular Diagnostics, National Centre of Excellence in Molecular Biology, University of the Punjab, Lahore 53700, Pakistan. Email: idreeskhan@cemb.edu.pk

only the HCV genotype dominant in the population.[6,7] As a result, genotypes present at lower proportional levels in a mix could be missed or mistyped.[8]

To overcome these problems a new genotyping method based on primer-specific polymerase chain reaction (PCR)[9] followed by polyacrylamide gel electrophoresis (PAGE) and two currently used genotyping assays, restriction fragment length polymorphism (RFLP) analysis[10] and Murex HCV serotyping (Murex, Burgwedel, Germany) 1–6 assay was assessed for its sensitivity and reliability for detection of mixed genotypes.

MATERIALS AND METHODS

Source of samples

Overall 20 samples with mixed genotypes such as 1a + 1b ($n = 4$), 1a + 3a ($n = 2$), 3a + 1b ($n = 3$), 3a + 3b ($n = 9$), 3a + 4 ($n = 2$) were used for the establishment of the procedure for the detection of HCV infection with mixed genotypes. In addition, positive plasma samples from 50 thalassemic patients that had received multiple transfusions in the past and were positive for anti-HCV antibody and serum HCV RNA were used to evaluate the assays further.

Genotyping PCR followed by PAGE

HCV genotyping PCR was carried out as described earlier with few modifications.[9] Briefly, HCV RNA was extracted from 100 μL serum sample using a Gentra RNA isolation kit (Puregene, Minneapolis, MN, USA). Of the total RNA solution, 100 U of Moloney murine leukemia virus reverse transcriptase enzyme (M-MLV RTE) (Life Technologies. Gaithersburg, MD, USA) was used for the reverse transcription of viral RNA to cDNA. An amplification of the 470-bp region from the synthesized cDNA (5′NCR and core region) was carried out in the first PCR round. Two second-rounds of PCR were performed for each sample using two different primer mixtures; one containing primers for the HCV genotypes 1a, 1b, 1c, 3a, 3c and 4 and another mix including the 2a, 2c, 3b, 5a and 6a primers. The genotype 3a-specific primer (5′-ACT CCACCAACGATCTGTCC-3′; nucleotide 441–422) used previously was replaced by another genotype 3a-specific primer (5′-CCCAGGACCGGCCTTCGC TC-3′; nucleotide 569–550) for the best separation of bands. The type-specific fragments were separated by PAGE. Two μL of second-round PCR product along with 2 μL distilled water and 1 μL of loading dye were electrophoresed on 12% non-denaturing PAGE in a Hoefer Scientific Apparatus (Amersham Pharmacia Biotech, Little Chalfont, UK) at 160 V for 1 h and was fixed in 7.5% glacial acetic acid solution. The gel was rinsed thrice with distilled water and added the staining solution (1% silver nitrate + 0.15% of the 37% formaldehyde). After 30 min the staining solution was discarded and the gel rinsed in distilled water for 10 s. The gel was placed in a pre-chilled developing solution (3% sodium carbonate + 0.15% of 37% formaldehyde + 2 μg/mL sodium thiosulfate) and agitated until the bands started to develop. The developing reaction was stopped by adding the pre-chilled 7.5% glacial acetic acid directly to the developing solution and agitated for 2 min. The gel was rinsed twice with distilled water, viewed on a white light box and photographed. A 100-bp DNA ladder (Life Technologies, USA) was run in each gel as a DNA size marker.

RFLP analysis for HCV genotyping

RFLP for HCV genotyping was performed as described previously by Furione *et al*.[10]

Murex HCV 1–6 serotyping

HCV serotyping was performed using Murex HCV Serotyping 1–6 assay kit (Murex, Burgwedel, Germany) according to the protocol given in the kit manual.

Assessment of sensitivity and specificity of the assays

To assess the sensitivity and reliability of our previously developed assay[9] and RFLP[10] method for detecting mixed genotypes in a viral population, an artificial mixing experiment was performed with a defined load of the HCV RNA of genotype 1a and 2a. Quantification of HCV RNA for the genotypes was performed on an AcuGen Minilyzer (Biotronics, Lowell, MA, USA) according to the manufacturer's instructions. A total of 45 μL of distilled water was added to five tubes labeled 10^5, 10^4, 10^3, 10^2 and 10, respectively. The dilution series was prepared by adding 5 μL of each tube to the next tube and mixing it between each transfer. The dilution factor was 10. The RNA was mixed in the following proportions of genotype 2a to genotype 1a (IU/mL): 10^6:10^6 (50:50%), 10^6:10^5 (58.3:41.6%), 10^6:10^4 (66.6:33.3%), 10^6:10^3 (75:25%), 10^6:10^2 (83.4:16.6%) and 10^6:10 (91.6:8.3%). To detect mixed genotypes in a viral population, an artificial

mixing experiment was similarly performed with defined load of HCV RNA of genotype 1a and 1b.

RESULTS

Sensitivity and reliability of the assay

Figure 1 shows that 8.3% of the genotype in the mix can be clearly detected by our previously developed method. RFLP analysis was able to detect a genotype at a level of 41.6% or above in a mixed-genotype population. Any genotype at levels below 25% could not even be identified by direct DNA sequencing. This shows an approximate threefold increase in the sensitivity of this assay over that of direct DNA sequencing for the detection of mixed genotypes. The present system easily detects infections with mixed genotypes

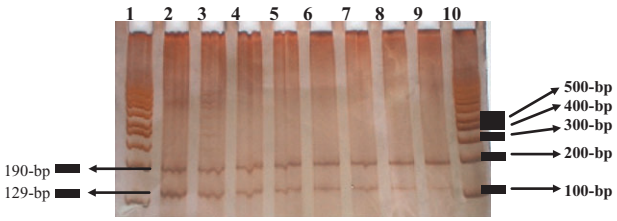

Figure 1. Typical photograph of polyacrylamide gel electro-phoresis of polymerase chain reaction products showing genotype 1a (129 bp) and 2a (190 bp) detection at a level of 8.3% in a mixture of two genotypes after artificial mixing performed by our reported method. Lanes 1 and 10 show 100 bp DNA size marker. An artificial mixing experiment was performed with defined load of hepatitis C virus RNA of genotype 1a and 2a. The dilution factor was 10. The RNA was mixed in the following proportions of genotype 2a to genotype 1a: 106:106 (50:50%) lanes 2-3; 106:105 (58.3:41.6%) lane 4; 106:104 (66.6:33.3%) lane 5; 106:103 (75:25%) lane 6; 106:102 (83.4:16.6%) lane 7; and 106:10 (91.6:8.3%) lanes 8-9.

2a and 1a; 3a and 3b; and 1a and 1b due to the different sizes of type-specific PCR bands in an artificial mixing experiment.

Comparison of sensitivities of new assay, RFLP analysis method and serotyping assay for the detection of HCV mixed-genotype infections

A total of 50 HCV isolates from thalassemic patients who had received multiple blood transfusions were genotyped and analyzed for a comparative study of the sensitivity of the three genotyping methods. Table 1 summarizes the genotyping results generated by all the three methods. Out of total 50 HCV isolates 21 were found with mixed genotypes by the newly developed genotyping method. The serotyping and RFLP methods detected mixed genotypes in only nine and five HCV isolates, respectively. The results showed that viruses in approximately 42% of the samples from this group were determined to be infected with mixed genotypes using this new method. The serotyping assay and RFLP analysis performed poorly, being able to identify only 18% and 10% of mixed-genotype infections, respectively. Of the 21 mixed genotype cases identified by our new method, nine contained mixed genotypes 3a + 3b, five contained 1a + 3a, three contained 1a + 3b, two contained 1a + 2a and one each contained 3a + 4 and 1a + 1b. Table 2 further summarizes the concordances and discordances of the three evaluated methods.

DISCUSSION

The reliable detection of HCV mixed-genotype infections may have broad applications and can be used to: (i) gain a precise understanding of the mechanism of genotype changes over time;[11] (ii) facilitate studies of the clinical and biological differences among HCV

Table 1. Genotyping results of hepatitis C virus (HCV) genotypes generated by all the three methods in thalassemic patients ($N = 50$)

HCV genotype	New genotyping†	RFLP‡	Serotyping§
1	5 (1a = 2, 1b = 3)	5 (1a = 3, 1b = 2)	7
2	2 (both 2a)	2 (both 2a)	2
3	20 (3a = 18; 3b = 2)	32 (3a = 25; 3b = 7)	28
4	1	1	2
Mixed	21	9	5
Not typed	1	1	6
Total	50	50	50

†Performed with new genotyping assay using polyacrylamide gel electrophoresis instead of agarose gel electrophoresis (Idrees[9]).
‡Restriction fragment length polymorphism (RFLP) for HCV genotyping was carried out as described previously by Furione *et al.*[10]
§HCV serotyping was done using Murex HCV Serotyping 1–6 Assay according to the protocol given in the kit manual.

Table 2. Discrepancies among the three assays for mixed-genotypes detection (*N*= 50) of hepatitis C virus (HCV) genotypes

HCV genotype	New system[†]	RFLP[‡]	Serotyping[§]	Comments
3a + 3b	9	3	–	Serotyping cannot differentiate between 3a and 3b and RFLP is a less sensitive method as only three out of nine from this category were typed.
1a + 3a	5	1	6 (type 1 + 3)	RFLP is less sensitive
1a + 3b	3	0	0	Only one genotype (1a) for two samples by RFLP and serotyping assays (1) and the third sample was not typed by both methods.
1a + 2a	2	0	1 (type 1 + 2)	Designated type 1 + 2 for the first sample and only type 2 for the second samples by serotyping assays. Neither sample was typed by the RFLP method.
3a + 4	1	1	1	Concordance among all assays
1a + 1b	1	0	–	Serotyping cannot differentiate between 1a and 1b
1 + 4	0	0	1	This sample was genotype 3a, by the new system and the RFLP method.
Total	21	5	9	Serotyping assay and RFLP analysis performed poorly, being able to identify only 18% and 10% of mixed-genotype infections, respectively, compared to 42% mixed-genotype infection designated by the new system.

[†]Genotyping was performed with new genotyping assay using polyacrylamide gel electrophoresis instead of agarose gel electrophoresis (Idrees[9]). [‡]Restriction fragment length polymorphism (RFLP) for HCV genotyping was performed as described previously by Furione *et al.*[10] [§]HCV serotyping was performed using Murex HCV Serotyping 1–6 Assay, according to the protocol given in the kit manual.

genotypes in which confounding host factors could be completely eliminated;[12] and (iii) use as a marker for monitoring the presence of risk factors in a population.[13] Though most current genotyping methods are reliable[7] for the general genotyping of HCV, none of these methods is suitable for detecting mixed genotypes.[6] Population-based DNA sequencing is the only reliable method for detecting mixed-genotype infections, however, it is not practical for large cohort studies since it is expensive, time-consuming and requires expertise. Therefore we assessed our previously developed HCV genotyping method using type-specific PCR, but used PAGE instead of the previously used agarose gel,[9] and RFLP and serotyping assays for their sensitivity and reliability in detecting infections with mixed HCV genotypes.

Several interesting findings emerged from the present study. The most interesting is the higher sensitivity of this newly developed method for detecting HCV mixed-genotype infections because it was able to detect a genotype present at levels as low as 8.3% in a defined mix of HCV genotypes, showing an approximately threefold increase in sensitivity over that of direct DNA sequencing. Previously genotype 1a present at levels below 25% was not identified by direct DNA sequencing.[8] This method was a more than five times as sensitive as RFLP analysis, as the latter was able to detect a genotype at the level of 41.6% or above in a mixed-genotype population. Due to this increased sensitivity it has the potential to estimate more accurately the prevalence of mixed-genotype infections in different populations, which may have important implications for clinical and epidemiological studies.

A limitation seen in most current genotyping assays in use (based on the HCV 5′UTR region) is the identification of mixed infections with genotypes 1a and 1b, as the only single nucleotide difference at position 99 is available to distinguish between the two genotypes.[13] Interestingly, this limitation is not applicable in our developed genotyping method as we were able to detect infections with mixed genotypes 1a and 1b easily due to different sizes of type-specific PCR bands that were based on the 5′UTR region and core gene. It is important to note here that genotypes 1a and 1b are the most prevalent HCV genotypes circulating in many parts of the world; therefore, this system is eligible for the detection of true mixed-genotype infections with 1a and 1b genotypes.

Most importantly, the results of the present study show a clear picture of the true prevalence of HCV mixed-genotype infections in Pakistan, which is higher than that estimated previously by the widely used genotyping methods with samples from blood donors and patients with chronic hepatitis C.[4,11] Therefore, this newly developed method may be an alternative for the accurate detection of HCV

mixed-genotype infections including multiple-genotype infections in large cohort studies.

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REFERENCES

1 Ogata N, Alter HJ, Miller RH, Purcell RH. Nucleotide sequence and mutation rate of the H strain of hepatitis C virus. *Proc Natl Acad Sci U S A* 1991; **88**: 3392–6.
2 Martell M, Esteban JI, Quer J, Genesca J, Weiner A, Gomez J. Hepatitis C virus circulates as a population of different but closely related genomes: quasispecies nature of HCV genome distribution. *J Virol* 1992; **66**: 3225–9.
3 Martial J, Morice Y, Abel S *et al*. Hepatitis C virus (HCV) genotypes in the Caribbean island of Martinique: evidence for a large radiation of HCV-2 and for a recent introduction from Europe of HCV-4. *J Clin Microbiol* 2004; **42**: 784–91.
4 Jarvis LM, Ludlam CA, Simmonds P. Hepatitis C virus genotypes in multi-transfused individuals. *Haemophilia* 1995; **1** (Suppl): 3–7.
5 Forns X, Maluenda MD, Lopez-Labrador FX *et al*. Comparative study of three methods for genotyping hepatitis C virus strains in samples from Spanish patients. *J Clin Microbiol* 1996; **34**: 2516–252.
6 Eyster ME, Sherman KE, Goedert JJ *et al*. Prevalence and changes in hepatitis C virus genotypes among multitransfused persons with hemophilia. The Multicenter Hemophilia Cohort Study. *J Infect Dis* 1999; **179**: 1062–9.
7 Lau JY, Mizokami M, Kolberg JA *et al*. Application of six hepatitis C virus genotyping systems to sera from chronic hepatitis C patients in the United States. *J Infect Dis* 1995; **171**: 281–9.
8 Hu YW, Balaskas E, Kessler G *et al*. Primer specific and mispair extension analysis (PSMEA) as a simple approach to fast genotyping. *Nucleic Acids Res* 1998; **26**: 5013–5.
9 Idrees M. Development of an improved genotyping assay for the detection of hepatitis C virus genotypes and subtypes in Pakistan. *J Virol Methods* 2008; **150**: 50–6.
10 Furione M, Simoncini L, Gatti M *et al*. HCV genotyping by three methods: analysis of discordant results based on sequencing. *J Clin Virol* 1999; **13**: 121–30.
11 Tuveri R, Rothschild C, Pol S *et al*. Hepatitis C virus genotypes in French haemophiliacs: kinetics and reappraisal of mixed infections. *J Med Virol* 1997; **51**: 36–41.
12 Horiike N, Masumoto T, Michitaka K *et al*. Response to interferon in chronic hepatitis C due to mixed genotype infection. *J Gastroenterol Hepatol* 1996; **11**: 353–7.
13 Hu Y, Balasas E, Furione M *et al*. Comparison and application of a novel genotyping method, semiautomated primer-specific and mispair extension analysis, and four other genotyping assays for detection of hepatitis C virus mixed genotype infections. *J Clin Microbiol* 2000; **38**: 2807–13.