Concordance of Hepatitis C Virus Subtyping by Non-structural 5A and Non-structural 5B Sequencing

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ABSTRACT

The non-structural 5B (NS5B) gene is the target region to identify hepatitis C virus (HCV) subtypes. However, it is not always possible to amplify this region because of inherently high sequence variability. Nucleotide sequences of the non-structural 5A (NS5A) and NS5B genes and its concordance were determined from patients infected with HCV genotype 1 (HCV-1). Among the 30 HCV-1 samples, 7 (23%) were identified as subtype 1a and 23 (77%) were identified as 1b by NS5A sequencing. Sequence analysis of the NS5B showed that 13 (43%) were identified as 1a and 17 (57%) were identified as 1b. Out of the 13 samples identified as 1a by NS5B, 6 (46%) were correctly identified by NS5A. Of the 17 samples identified as 1b by NS5B, 16 (94%) were correctly identified by NS5A. The presence of glutamic acid (E) or aspartic acid (D) at position 2225 in the NS5A differentiates 1a from 1b subtypes, respectively. This study showed that the NS5A sequencing can identify HCV-1a and 1b subtypes with predictive values of 86% and 70% of cases, respectively. The overall concordance with NS5B was 73%. NS5B sequence analysis remains to be the reference method to identify HCV-1 subtypes. NS5A sequencing may be used to complement NS5B sequencing in case the NS5B gene cannot be successfully amplified.

Key Words: Hepatitis C virus, subtyping, non-structural 5A, non-structural 5B, DNA sequencing

Introduction

Hepatitis C virus (HCV) infection is a global health problem with an estimated 170 million chronic carriers worldwide. Six major genotypes and more than 90 subtypes have been identified worldwide. The HCV genotypes vary in different geographical regions. Genotype 1 is distributed worldwide and is predominant in Europe and the United States. Genotype 2 represents 10% to 30% of HCV genotypes and is particularly common in Japan and Italy. Genotype 3 is prevalent among intravenous drug users in Europe and the United States. Genotypes 4 and 5 are essentially restricted to the Middle East and South Africa, respectively. Genotype 6 seems to be confined in Southeast Asia particularly in Hong Kong and Vietnam.[1,2,3,4,5] In the Philippines, a vast majority of HCV samples were found to be either subtype 1a or 1b.[6,7,8,9] HCV creates a huge disease burden, since it accounts for 20% to 30% of cases of acute hepatitis, 70% to 80% of cases of chronic hepatitis, 40% of cases of end-stage liver disease, 50% to 76% of cases of hepatocellular carcinoma (HCC) and 30% to 40% of liver transplants.[10,11,12]

HCV is a positive strand RNA virus that has been classified in the genus *Hepacivirus* of the family *Flaviviridae*. The positive sense RNA genome is translated to produce a polyprotein which is processed to generate the mature structural and non-structural proteins.[13,14] Analysis of the nucleotide sequence homology of various hepatitis C virus genomic regions such as the 5'untranslated region (5'UTR) and regions coding for the envelope (E1), core, and non-structural 5B (NS5B) led to the identification of six (6) major genotypes, formerly denoted as clades, and numerous closely related subtypes within the genotypes.[15,16] The NS5B region sequence analysis is considered the reference method and has been recommended for HCV genotyping and subtyping in consensus proposals.[17,18] However, it has been shown that it is not always possible to amplify this region because of primer-target mismatch within the highly variable NS5B sequence. In addition, failure in sequencing the NS5B gene has been reported by some investigators despite successive attempts to amplify cDNA with the NS5B primers.[19,20] The present study evaluated the accuracy of subtyping of HCV-1 samples by partial NS5A sequencing as compared with the partial NS5B sequence.

This basic knowledge on HCV genomic heterogeneity has clinical implications in prognostication as well as treatment. The prognostic significance lies in the association of specific genotypes and subtypes with severe liver disease such as chronic hepatitis, cirrhosis and HCC. Moreover, some genotypes and subtypes has been shown to be resistant to antiviral treatment, with HCV genotypes 2 and 3 being associated with better response, compared with genotype 1. Marto *et al.*, (2008) found that the identification of the infecting genotype and subtype is an

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important tool to optimize its treatment type, its duration of therapy and its correct dose.[18]

Methods

Patients

Thirty (30) blood samples from patients infected with HCV-1, previously confirmed by PCR-RFLP and clinically diagnosed with chronic hepatitis C, collected from May 2005 to December 2008 at St. Luke's Medical Center, Philippines were analyzed. There were 17 males and 13 females with ages ranging from 32 to 76 years old. Patients are excluded if they have other causes of liver diseases such as autoimmune hepatitis and history of alcoholism, and if they were found to be reactive to hepatitis B surface antigen (HBsAg) and antibody to hepatitis B core antigen (anti-HBc).

HCV RNA Extraction

The viral RNA from HCV-infected patient plasma was extracted from peripheral blood using the QIAamp® Viral RNA Mini kit from Qiagen according to manufacturer's instructions. Briefly, 140 µl of plasma was digested at 56ºC for 5 minutes with AVL buffer as provided by the kit. Absolute ethanol was added for the purpose of precipitation and the mixture was transferred to the spin column and washed three times using the provided buffer to purify the viral RNA that binds to the column. The viral RNA was then eluted and collected from the column. The nucleic acid concentration and purity was determined by measuring the absorbance at 260/280 nm using the NanoDrop® 1000 Spectrophotometer (Thermo Fisher Scientific).

cDNA Synthesis

Reverse transcription was carried out from 10 µl of viral RNA extract using the SuperScript™ III reverse transcriptase (Invitrogen™).

NS5A Nested PCR Amplification

The NS5A amplification was carried out with 0.25 pmol of outer sense primer 5′-CAGTGCTCACTTCCATGCTCA-3′; and outer antisense primer 5′-ACGGATATTTCCCTCTCATCC-3′, 0.02 U Phusion™ DNA polymerase, 0.2 mM dNTPs, 1X Phusion™ HF buffer, and PCR-grade water with the following thermal profile: initial denaturation at 98°C for 30 seconds followed by 35 cycles at 98°C for 10 seconds, 56°C for 10 seconds, 72°C for 30 seconds, and final extension at 72°C for 10 minutes.[21] The conditions of the second PCR were the same as described above using inner pair of sense and antisense primers 5′-ACCCCTCCCACATTACAGCAG-3′ and 5′-CCGAAGCGGATCGAAAGAGTCCA-3′.

NS5B Nested PCR Amplification

The NS5B amplification was carried out with 0.5 pmol of outer sense primer 5′-TGGGGTTCTCGTATGATACCC-3′ and outer antisense primer 5′-CCTGGTCATAGCCTCCGTGAA-3′, 0.02 U Phusion™ DNA polymerase, 0.2 mM dNTPs, 1X Phusion™ HF buffer, and PCR-grade water with the following thermal profile: initial denaturation at 95°C for 1 minute, followed by 40 cycles at 95°C for 20 seconds, 56°C for 30 seconds, 72°C for 1 minute, and final extension at 72°C for 10 minutes.[22] The conditions of the second PCR were the same as described above using inner pair of sense and antisense primers 5′-GATACCCGCTGCTTTGACTC-3′and 5′-CCTCCGTGAAGGCTCTCAG-3′.

DNA Purification and Nucleotide Sequencing of the NS5A and NS5B

The nested PCR products were purified using the GFX™ PCR DNA and Gel Band Purification kit (GE Healthcare). Amplicons in the NS5A and NS5B regions were sequenced using Big Dye Terminator Cycle Sequencing Ready Reaction Kit and Applied Biosystem 3730xl automated sequencer (Macrogen, Korea). Sequence data were aligned with the consensus sequences of known subtypes using bioinformatics tools such as ChromasPro version 1.34 and BioEdit version 7.0. The nucleotide sequences were compared for identity with sequence from the National Center for Biotechnology Information (NCBI) using the basic local alignment search tool program (BLAST). The GenBank/EMBL/DDBJ accession numbers of HCV sequences used in the analysis were M62321 (HCV-1) for subtype 1a, D90208 (HCV-J) for subtype 1b. Biosafety and quality control measures were observed throughout the conduct of this study.

Results

Nucleotide sequencing of the NS5A revealed that 7 (23%) and 23 (77%) of the 30 samples were identified as subtypes 1a and 1b, respectively. In contrast, sequence analysis of the NS5B showed that 13 (43%) were identified as 1a and the remaining 17 (57%) were identified as 1b (Table 1). Out of the 13 samples identified as 1a by NS5B, 6 (46%) were correctly identified by NS5A. Of the 17 samples identified as 1b by NS5B, 16 (94%) were correctly identified by NS5A. The predictive value of NS5A to subtype 1a was 6/7 (86%). For subtype 1b, the predictive value of NS5A was 16/23 (70%). The overall concordance between NS5A and NS5B was 73% (Table 2).

Table 1. Genomic identification of HCV-1 subtypes by partial NS5A and NS5B sequencing.

	SEQUENCING	
	NS5A	NS5B
1a	7 (23%)	13 (43%)
1b	23 (77%)	17 (57%)
TOTAL	30	30

Table 2. Concordance of HCV-1 subtyping by NS5A and NS5B sequencing.

		NS5B Subtyping		
		1a	1b	Total
NS5A sequencing	1a	6	1*	7
	1b	7*	16	23
	Total	13	17	30

The NS5A sequencing is horizontally indicated and the NS5B is presented vertically.

* Inconsistent subtype assignment.

In this study, it was shown that HCV-1a viruses always presented with one substitution, glutamic acid (E) at position 2225, as compared to HCV-1b reference sequence (Table 3). While HCV-1b viruses always presented with one substitution, aspartic acid (D) at position 2225, with respect to the HCV-1a reference sequence (Figures 1 and 2).

Table 3. Identification of key amino acids in the NS5A gene.

	D2225	E2225	Total
1a	0 (0%)	7 (100%)	7
1b	23 (100%)	0 (0%)	23
Total	23	7	30

Discussion

Accurate identification of HCV genotypes and subtypes has important clinical and epidemiological implications.[23] Until now, the non-structural 5B (NS5B) is the preferred region for both genotyping and subtyping, and has been recommended in consensus proposals. However, it has been shown that it is not always possible to amplify this region because of lack of conservation in the primer-binding sites. Our study evaluated the accuracy of subtyping of HCV-1 samples by partial NS5A sequencing compared with the partial NS5B sequence.

The present study showed that the NS5A sequencing can identify subtypes 1a and 1b with predictive values of 86% and 70% of cases, respectively. Overall, among the 30 HCV-1 samples, 22 (73%) were concordantly subtyped by NS5A sequencing (Tables 1 and 2). The non-structural 5 gene was chosen because this region is sufficiently variable, and could be readily amplified from plasma of HCV-infected individuals and because there is already considerable quantity of published sequence data in this region. Nucleotide sequencing of a subgenomic region is preferred to identify HCV subtypes because full length sequence analysis is time-consuming, expensive and impractical in a clinical setting. This is based on the assumption that a particular subgenomic region is representative of the entire viral genome.[16] In 2007, Ross *et al*., reported that this supposition is still valid because both inter-genotypic and inter-subtype recombinations of HCV are rare events.[23] In addition, it has been suggested that with the exception of the 5'untranslated region (5'UTR), any region of the genome can be used as the basis for virus subtype identification, provided sequences from major databases such as the GenBank, European Molecular Biology Laboratory (EMBL), and DNA Databank of Japan (DDBJ) are available.[24]

Our study demonstrated that glutamic acid (E) at position 2225 was present in 7/7 (100%) subtype 1a viruses, while aspartic acid (D) at position 2225 was present in 23/23 (100%) subtype 1b viruses, a change that can be considered subtype specific (Table 3). Results suggest that glutamic acid (E) at position 2225 can be used as a marker to reliably identify HCV-1a subtypes. On the other hand, aspartic acid (D) at position 2225 in the NS5A region of the HCV genome can be used as a marker to reliably identify HCV-1b subtypes. Recently, it has been reported that alignments obtained with sequences from databases confirmed that glutamic acid (E) at position 2225 are widely distributed in HCV-1a subtype, while aspartic acid

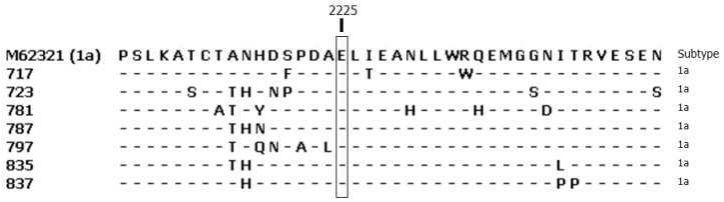

Figure 1. Deduced amino acid alignment of NS5A of 7 HCV-1a confirmed samples. At the top, HCV-1a reference sequence (GenBank accession number M62321) is given. At the left, the sample identification numbers are shown. The key amino acid associated with 1a is boxed.

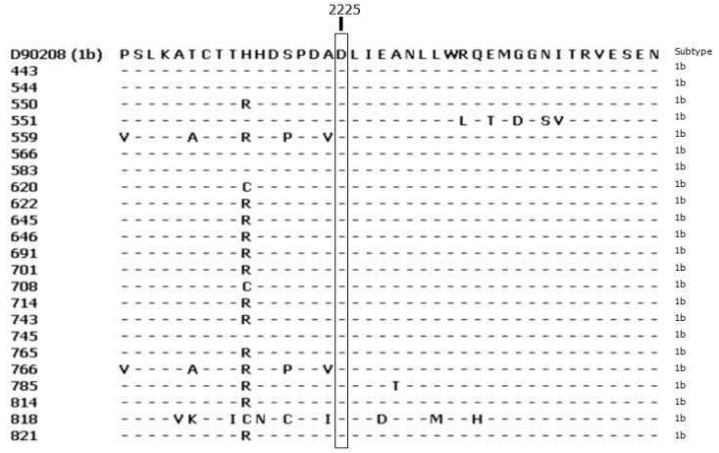

Figure 2. Deduced amino acid alignment of NS5A of 23 HCV-1b confirmed samples. At the top, HCV-1b reference sequence (GenBank accession number D90208) is given. At the left, the sample identification numbers are shown. The key amino acid associated with 1b is boxed.

(D) at position 2225 are widely distributed in HCV-1b subtype and thus, can be considered as 1a and 1b markers. This study corroborates with the findings of Torres-Punte *et al*., (2008) showing that glutamic acid (E) and aspartic acid (D) are distributed worldwide among HCV-1a and 1b viruses and thus, could be considered subtype-specific.[25] In addition, results suggest that sequence analysis of variable regions of the HCV genome, specifically the NS5A, spanning from nucleotides 6954 to 7073 may be used as an alternative target region for identification of HCV-1 subtypes.

Recently, it has been reported that there is a slight difference in treatment outcomes between HCV-1a and 1b-infected patients.[12] HCV-1b for example, is associated with less favorable prognosis following antiviral treatment.[26] In addition, based on a local study by Barzaga *et al*., (1994), certain subtypes appear to be more responsive to interferon therapy. Thus, accurate typing is an indispensable tool for tailoring antiviral treatment as well as in epidemiological investigations.[27]

Overall, these findings have major implications for epidemiologic HCV investigations as to its origin and spread of infection, its distribution, its routes of transmission including outbreak studies and novel transmission risks, its association with certain risk groups and for viral evolutionary studies.

It has been shown that patients infected with subtype 1b have been found to have more advanced liver disease, which may suggest increased pathogenicity. Thus, accurate subtyping may later on help in decision making for clinical management of HCV infection. In addition, molecular characterization of HCV-1 subtypes is likely to facilitate and contribute to the development of an effective vaccine against infection with HCV.

Conclusion

NS5A sequencing can identify HCV-1a and 1b subtypes with predictive values of 86% and 70% of cases, respectively. The overall concordance with NS5B was 73%. NS5B sequence analysis remains to be the reference method to identify HCV-1 subtypes. NS5A sequencing may be used to complement NS5B sequencing in case the NS5B gene cannot be successfully amplified.

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Leading Causes of New Patient Consults at the Out-Patient General Eye Clinic of the Sentro Oftalmologico Jose Rizal, Philippine General Hospital

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ABSTRACT

Objective. To determine the current leading causes of new patient consults at the out-patient general clinic of the Department of Ophthalmology and Visual Sciences (DOVS), Sentro Oftalmologico Jose Rizal (SOJR).

Methods. The data were gathered from the DOVS out-patient general clinic monthly census. Compilation and tabulation of the diagnoses of all new patients from January to December 2009 were done.

Results. The leading causes of new patient consults were cataract (30.8%), error of refraction (20.1%), pterygium (6.1%), conjunctivitis (4.9%), dysfunctional tear syndrome (4.5%), glaucoma (4.3%), diabetic retinopathy (3.7%), and hypertensive retinopathy (3.4%).

Conclusion. Cataract and error of refraction comprise the majority of all causes of consultation among new patients.

Key Words: cataract, error of refraction

Introduction

Blindness remains to be a public health problem in developing countries according to the World Health Organization (WHO).[1] The global initiative of the WHO is to bring down the prevalence of blindness. Results of the most recent national survey of blindness in the Philippines reported that cataract is the most common cause of blindness and error of refraction is the most common cause of low vision.[2]

The Philippine General Hospital (PGH) is considered to be the largest government hospital with the most number of patient consultations in the country. The DOVS of the PGH transferred from ward 12 of the main hospital building to its present location at the SOJR starting in 2005. The SOJR is a state of the art facility donated by the Kingdom of Spain to help address the problem of blindness. The number of patients served by the DOVS greatly increased ever since the relocation to the SOJR.[3]

A search of the local literature regarding causes of visual complaints among patients consulting for the first time at the out-patient eye clinic (new patient) in the PGH revealed three articles which were reported in 1923,[4] 1953[5] and 1971.[6] Since then, there has been no additional information published.

This study aimed to determine the current leading causes of new patient consults at the out-patient general clinic of the DOVS, SOJR, PGH.

Methods

This retrospective study was a compilation of the new patient monthly census at the DOVS, PGH out-patient general clinic. The new patient monthly census included the first consult diagnosis directly responsible for the patient's chief complaint. When two or more diseases were involved, these co-existing diseases were taken into consideration and recorded. We compiled and tabulated the diagnoses of all new patients recorded from January to December 2009.

In 2006, the DOVS transferred all its services to the SOJR. The data from the annual reports of the DOVS from 2006 to 2008 were compared.[7]

Results

A total of 10,750 new patient consults from the out-patient general clinic of the DOVS, SOJR, PGH were seen in 2009. Table 1 shows the leading causes of new patient consults. Cataract was the most common condition and comprised 30.8% of all cases. Error of refraction was second accounting for 20.1%. Completing the top five causes were pterygium (6.1%), conjunctivitis (4.9%) and dysfunctional tear syndrome (4.5%). The rest of the leading causes were glaucoma (4.3%), diabetic retinopathy (3.7%), and hypertensive retinopathy (3.4%).

Table 2 shows the leading causes of new patient consults in 2009 compared to 2008, 2007 and 2006. The leading causes from 2006 to 2008 were generally similar compared to 2009. Cataract was also the number one cause followed by error of refraction.

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Table1. Leading causes of new patient consults in 2009

Causes	Number of consults	Percent
1. Cataract	3,305	30.8
2. Error of refraction	2,219	20.1
3. Pterygium	655	6.1
4. Conjunctivitis	526	4.9
5. Dysfunctional tear syndrome	471	4.5
6. Glaucoma	456	4.3
7. Diabetic retinopathy	385	3.7
8. Hypertensive retinopathy	366	3.4
Others	2,367	22.2
Total	10,750	100

Table 2. Leading causes (percent) of new patient consults in 2009 compared to 2008, 2007 and 2006

Causes	2009 (%)	2008 (%)	2007 (%)	2006 (%)
1. Cataract	30.8	36.8	29.6	29.1
2. Error of refraction	20.1	8.1	8.5	6.7
3. Pterygium	6.1	5.6	5.4	5.8
4. Conjunctivitis	4.9	4.6	4.5	2.1
5. Dysfunctional tear syndrome	4.5	2.5	2.6	2.6
6. Glaucoma	4.3	3.8	3.3	2.8
7. Diabetic retinopathy	3.7	2.8	2.7	1.7
8. Hypertensive retinopathy	3.4	2.7	2.7	2.1
Others	22.2	33.1	40.7	47.1
Total	100	100	100	100

Table 3. Leading causes (percent) of new patient consults in 2009 compared to 1968-70, 1952-53 and 1918-29

Causes	2009(%)	1968-70(%)	1952-53(%)	1918-29(%)
1. Cataract	30.7	8.8	7.4	3.8
2. Error of refraction	20.1	27.2	12	5
3. Pterygium	6.1	4.7	5	NA
3. Conjunctivitis	4.9	14.6	13	21
5. Dysfunctional tear syndrome	4.5	NA	NA	NA
6. Glaucoma	4.3	1.2	1.7	0.9
7. Diabetic retinopathy	3.7	0.2	NA	NA
8. Hypertensive retinopathy	3.4	0.2	NA	NA

NA – no actual data was reported because cases were significantly few

Discussion

The WHO in 2002 reported that the number of people with visual impairment worldwide was estimated to be in excess of 161 million.[5] More than 90% of visually impaired people live in developing countries. Cataract is responsible for 48% of the world's blindness.

In 2009, cataract was the leading cause of new patient consults seen in the DOVS, SOJR, PGH. The second most common cause was error of refraction. Cataract (30.8%) and error of refraction (20.1%) when combined formed the majority (50.9%) of new patient eye consults. Other causes had a comparatively lower incidence. These were anterior segment eye diseases which included pterygium, conjunctivitis, and dysfunctional tear syndrome. Posterior segment eye diseases such as glaucoma, diabetic retinopathy, and hypertensive retinopathy followed.

Table 2 reveals that cataract was consistently the most frequent cause of new patient consults from 2006 to 2009. Cataract comprised about thirty percent of all new patient consults for the past four years. Error of refraction was also consistently the second most common cause. The recent opening of an optical shop in the SOJR resulted in a three-fold increase in 2009 of patients consulting with error of refraction. Pterygium and conjunctivitis were usually in the top five causes. A significant increase of dysfunctional tear syndrome in 2009 was noted compared to the previous years. This may be attributed to the setting up of a separate clinic for dry eye patients. Glaucoma, diabetic retinopathy and hypertensive retinopathy showed an increasing trend.

In previous literature, Fernando reported that conjunctivitis was the leading cause of new patient consults in 1918-29[6] and 1952-53[7] (Table 3). In 1968-70, Mangubat reported that error of refraction significantly increased and was the most frequent cause.[8] Our report shows that cataract followed by error of refraction are currently the most common causes of eye consultations among new patients in PGH. There has been a shift from infectious causes to error of refraction to cataract through the years. The main reasons are the growing elderly population, the greater demand for perfect vision because of more demanding daily visual tasks, and the advances in cataract surgery and refractive procedures. Dysfunctional tear syndrome, glaucoma, diabetic retinopathy and hypertensive retinopathy consultations have been steadily increasing because of the availability of early detection and screening procedures as well as the growing public awareness of these eye diseases.

This study about the current leading causes of new patient consults at the out-patient general clinic of the SOJR echoes the results of the national survey of blindness that cataract is the most common cause of blindness and error of refraction is the most common cause of low vision. It also reveals that eye diseases seen in the PGH is a microcosm of the visual problems in the entire Philippines. Visual impairment due to cataract and error of refraction are remediable. Blindness and visual disability when detected and treated early can be prevented. Therefore, the cataract and refraction services must remain an important priority of the SOJR in its commitment in providing excellent eye care. The DOVS should actively continue its efforts to implement

the cataract backlog programs and the prevention of blindness activities of the Department of Health in coordination with the WHO Vision 20/20 global strategy to eliminate the avoidable causes of blindness by the year 2020.[9]

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