GENOTYPIC ANALYSIS OF HEPATITIS C VIRUS IN BLOOD DONORS IN INDONESIA

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Abstract. A study was conducted to describe the genetic diversity of hepatitis C virus (HCV) in a population of positive blood donors from throughout Indonesia. Repeat analysis by reverse transcription-polymerase chain reaction (RT-PCR) of 102 anti-HCV positive samples showed that 67 gave HCV-specific positive signals by the PCR for the 5′-untranslated genomic region of HCV. Further genotypic analysis on 64 HCV RNA-positive samples indicated that 57 belonged to the following individual genotypes: 1a, 1b, 2a, 2b, and 3b. The predominant HCV genotypes in this donor population were 1b (57.8 %), 2a (17.2 %), and 3b (10.9 %). The core sequences of the 4 indeterminate samples when aligned with published sequences of various HCV genotypes showed a range of homology from 16.16% to 78.67%. Comparative analysis of genotypic representation from other anti-HCV-positive study populations, including polytransfused pediatric and adult renal dialysis groups, is now being carried out to determine the potential genotypic association with mechanistic HCV spread.
A study of the identification of HCV genotypes and variants among HCV isolates from a select group of patients with acute hepatitis in a Jakarta, Indonesia hospital and in 3 patients with chronic liver disease has also been performed. However, it was reasoned that such data may be biased and reflect mostly HCV genotypes that have a high predilection for causing disease. The finding of a high prevalence of HCV 1b genotype in samples from the hospital-based study for causing disease. The finding of a high prevalence of acute hepatitis in a Jakarta, Indonesia hospital and in 3 patients with hepatitis supports this view. It was therefore reasoned that a study of the HCV genotypes and variants in a non-biased blood donor population may provide a more reasoned that such data may be biased and reflect mostly HCV genotypes that have a high predilection for causing disease. The finding of a high prevalence of HCV 1b genotype in samples from the hospital-based study for causing disease. The finding of a high prevalence of acute hepatitis in a Jakarta, Indonesia hospital and in 3 patients with hepatitis supports this view. It was therefore reasoned that a study of the HCV genotypes and variants in a non-biased blood donor population may provide a more objective analysis of the HCV genotypes and variants prevalent in the Indonesian archipelago. A study was thus conducted on the prevalence of HCV seropositivity among the blood donor population in Indonesia and the results are reported elsewhere. These HCV seropositive samples were then subsequently used to define HCV genotypes and variants. The results of the study of the genotypes and variants in this blood donor population constitute the basis of this report.

### MATERIALS AND METHODS

**Donor samples.** A total of 7572 blood specimens (6,732 males, 755 females, and 85 unknown) were collected during a survey of a total of 24 blood banks located in 21 of the 27 provinces that comprise the Indonesian archipelago. The collection of blood samples occurred from November 1992 to February 1993. All blood collection in Indonesia is totally voluntary and appropriate locally defined informed consent was duly acquired before obtaining each blood sample. The number of blood samples collected from each of the provinces was proportional to the population of the respective province. Collections were based on period specific activities, and not on any randomized sampling strategy. The mean ± SD age of the study population was 32.91 ± 10.02 years (range = 11–76 years). The mean ± SD age of the males was slightly higher (33.04 ± 4.07 versus 31.79 ± 10.48 years). No adverse effects of blood donation were noted. Standard epidemiologic data (emphasis on identifying standard blood-borne infection transmission risk factors) linked to each specimen collection were collected but without identifiers. All procedures were approved by the Human Investigation Committee of the Naval Medical Research Unit No. 2 prior to the initiation of the study.

**Serologic assays.** As stated earlier, all 7,572 serum samples were assayed for the presence of antibodies to HCV using a second-generation enzyme immunoassay (EIA) kit (Abbott Laboratories, North Chicago, IL) and the results of this study are reported elsewhere. Results showed that 161 of the 7,572 specimens were positive for antibodies to HCV.

**Polymerase chain reaction studies.** Serum samples from 102 of the 161 anti-HCV positive samples were available in sufficient volumes to allow for repeated analysis by RT-PCR and were thus further analyzed. Putative viral RNA extracted from these 102 specimens were first subjected to a PCR with primers encompassing the 5′-untranslated genomic region (5′-UTR) of HCV as previously described using the primer pairs identified as p32/p36 and p53/p48, resulting in 221-
and 126-basepair PCR products, respectively. Those samples showing positive results for HCV RNA were then subjected to genotyping with type-specific primers (Table 1) described elsewhere by Ohno and others. For those samples that were defined to be of indeterminate genotype with type-specific primers, the core sequences for each was amplified using universal primers, the amplified sequences were cloned and sequenced, and the new sequences were subjected to phylogenetic analysis. The techniques used for this are briefly described below.

**Extraction of viral RNA.** Viral RNA was extracted from 140 μl of serum using the QIAamp HCV kit (Qiagen, Inc., Hilden, Germany), followed by ethanol precipitation. This yielded a volume of 100 μl of RNA, of which 4 μl was used for reverse transcription.

**5'-UTR PCR.** cDNA was synthesized in a 10-μl reaction mixture containing 20 pmol of the outer sense primer (p36), 100 units of MMLV (Gibco-BRL, Gaithersburg, MD), 2 μl of 5× first-strand buffer (Gibco-BRL), 5 nmol of each dNTP (Takara, Inc., Tokyo, Japan), and 11 units of RNase inhibitor (Takara, Inc.). The mixture was incubated for 60 min at 42°C. It was then amplified in a first-round PCR (94°C for 1 min, 55°C for 1.5 min, and 72°C for 3 min) for 35 cycles in a 50-μl reaction mixture that contained 80 pmol of each dNTP, and 1.25 units of Taq polymerase (Takara, Inc.). The first-round PCR products were subjected to a second round of PCR (94°C for 1 min, 55°C for 1.5 min, and 72°C for 2 min) for 35 cycles in a 50-μl reaction mixture that contained 1 μl of the first-round PCR product, 40 pmol each of the anti-sense primer (p48) and the sense primer (p33), 10× PCR buffer, 2.5 nmol of each dNTP, and 1.25 units of Taq polymerase (Takara, Inc.).

**Genotyping.** For the genotyping studies, HCV type-specific primers designed by Ohno and others were used. Briefly, cDNA was synthesized using the universal outer anti-sense primer AC2 (10 pmol in a 10-μl reaction mixture) and the same ingredients as described above for the 5'-UTR PCR. The reaction mixture was incubated at 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min for 20 cycles. During the second-round PCR (94°C for 1 min, 62°C for 1 min, and 72°C for 1 min for 20 cycles), 2 different mixtures of anti-sense type-specific primers were included in the PCR mixture. Mixture 1 contained subtype-specific primers 1b, 2a, 2b, and 3b, whereas mixture 2 contained subtype-specific primers 1a, 3a, 4, 5a, and 6a. A primer concentration of 1 pmol was used for each anti-sense genotype-specific primer and the common primer. The other constituents were similar to those used for the 5'-UTR PCR. Universal primers were used to amplify undetermined HCV subtypes.

**Gel electrophoresis.** The PCR products were subjected to gel electrophoresis using a 2% or 3% NuSieve 3:1 agarose gel (FMC Bioproducts, Rockland, ME) for analysis of the 5'-UTR PCR and the genotyping PCR product, respectively. The electrophoresed bands were stained with 0.5 μg/ml of ethidium bromide (Merck, Inc., Darmstadt, Germany) and visualized with UV light. Genotype assignment was based on the relative length of the PCR product following the second-round PCR and use of type-specific primers. The primers used for genotyping are described in Table 1.

**Cloning and sequencing.** The PCR products that were
amplified using the universal primer were cloned into the PCR-11 vector using the TA cloning kit (Invitrogen, Inc., Groningen, The Netherlands). Plasmids derived were purified using the Qiagen Plasmid mini kit (Qiagen, Inc.) and sequenced.

Phylogenetic analysis. This analysis was carried out using the program DNADIST and NEIGHBOR provided with PHYLIP package designed by Felsenstein.

RESULTS

Antibodies to HCV were detected in 161 of the 7,572 blood samples, indicating a prevalence rate of 2.1% in the blood donor populations, as previously described. Of these 161 samples, 102 were available in sufficient quantities to allow repeat PCR analysis. Of these 102 samples analyzed, 67 (65.7%) gave HCV-specific positive signals by PCR of the 5'-UTR of HCV. Genotype analysis was performed on the HCV RNA-positive samples. Data obtained on the genotyping analysis of 64 of these samples using the type-specific primers outlined in the Materials and Methods are summarized in Table 2. Fifty-seven of the 64 samples analyzed appeared to be readily classified as those belonging to individual genotypes 1a, 1b, 2a, and 3b. Three samples appeared to contain 2 or more genotypes and 4 gave indeterminate results. Of the 57 that were readily classified into genotypes, 37 were typed as genotype 1b, 11 as genotype 2a, and 7 as genotype 3b. One sample each were typed as genotypes 1a and 2b. The predominant HCV genotype in this donor population were genotypes 1b, 2a, and 3b, accounting for 55 of the single genotype samples and 57 if one includes those samples with multiple genotypes. A typical electrophoretic pattern of the PCR products showing different genotypes is shown in Figure 1.

The 4 indeterminate HCV samples listed in Table 2 were sequenced as described in the Materials and Methods (samples NMR2A9–1, NMR2A42–1, NMR2A46–2, and NMR2A75–5), and their sequences aligned with the published sequences of various known HCV genotypes including 1a, 1b, 3 of 1c, 2a, 2b, 2c, 2d, 3a, 3b, 4a, 5a, 6a, 7a, 10a, and 11a. The core sequences of the indeterminate samples NMR2A9–1, NMR2A42–1, NMR2A46–2, and NMR2A75–5 are listed in Table 3. The comparative analysis of the indeterminate samples with previously reported genotype sequences is shown in Table 4. As seen, the degree of homology for each of the indeterminates varied from 95.16% to 78.67%.

It is important to note that the majority (86 of 102) of the samples were derived from individuals residing in the islands of Java and Bali. However, a few (16 of 102) samples were also available from the other islands of the Indonesian archipelago. Although the number is small, except for a single case of subtype 2b from a blood donor in Kalimantan, subtype 1b was identified in all other positive samples and no other subtypes were identified from outside the island of Java. This subtype was also the only subtype identified in positive samples from Sumatra, Kalimantan, Sulawesi, and eastern Indonesia. The subtype distribution within the Indonesian archipelago is shown in Figure 2.
Comparison of indeterminate with reported genotypes within a 310-basepair core sequence of hepatitis C virus

| Specimen No. NMR2A9-1 | GAATCTAAACCTCAAGGTACAACTACACCTAGTCCACGACAGGAGGACTTTAGGCTCTGCGGCTCAGCAGGTGATGTCGTCGTCGGTGGG | 81.29% |
| Specimen No. NMR2A9-2 | GAATCTAAACCTCAAGGTACAACTACACCTAGTCCACGACAGGAGGACTTTAGGCTCTGCGGCTCAGCAGGTGATGTCGTCGTCGGTGGG | 81.29% |
| Specimen No. NMR2A9-3 | GAATCTAAACCTCAAGGTACAACTACACCTAGTCCACGACAGGAGGACTTTAGGCTCTGCGGCTCAGCAGGTGATGTCGTCGTCGGTGGG | 79.68% |
| Specimen No. NMR2A9-4 | GAATCTAAACCTCAAGGTACAACTACACCTAGTCCACGACAGGAGGACTTTAGGCTCTGCGGCTCAGCAGGTGATGTCGTCGTCGGTGGG | 82.26% |

DISCUSSION

Previous studies on clinically diagnosed patients with hepatitis documented a prevalence rate of 44% HCV seropositive samples with genotype 1b. However, it was reasoned that these data may have been biased towards the HCV genotypes that cause the most clinically severe form of the disease and were not a true reflection of the genotypes and variants of HCV prevalent within the Indonesian archipelago. In efforts to provide a more non-biased sampling of the prevalence of HCV genotypes and variants in Indonesia, a study was conducted on blood bank donors. The results of these studies are reported herein. Several issues need to be addressed. First, the results of our data (Table 2) showed that similar to the prevalence of a high frequency of the 1b and 2a HCV genotypes (44% and 26%, respectively) in the patients with hepatitis, the 1b and 2a genotypes were present at high frequencies (57.8% and 17.2%, respectively) in the blood donor population in the studies reported herein. However, in contrast to the other genotypes, some notable differences exist. Thus, whereas HCV genotype 1c was significantly associated with clinically ill hepatitis patients (12%), the 3b HCV genotype was significantly prevalent (10.9%) in the blood donor population. The interpretation of these findings await more large-scale studies with a larger number of samples in efforts to determine whether these differences signify genotypes that induce disease and asymptomatic infections.

The pattern of distribution of the various HCV genotypes in the blood donor population in Indonesia basically reflects patterns observed in Asia. Analysis of data published appear to indicate that only 5 of the HCV genotypes (1a, 1b, 2a, 2b, and 3a) are prevalent in most parts of the world. Of these, genotype 1b is the one mostly associated with clinical disease and relatively refractory in its response to therapy with interferon-alpha. However, as alluded to earlier, it remains to be seen whether there are other genotypes that share this characteristic. Thus, continued efforts need to be made in identifying the various genotypes of HCV and the clinical-epidemiologic characteristics recorded to permit such an evaluation. It is also important to keep in mind that our criteria for classifying the genotypes may need to be reevaluated. Thus, of importance would be not only the sequence diversity data, but also the putative sequences that result in clinically relevant outcomes such as disease pathogenesis and those that may induce immune responses to prevent infection or at least disease. It is to be noted that the

| Specimen No. NMR2A9-2 | AAATCTCAAACTTCACAGACAAAACAACTAAACACACGCACCTACCACGACAGGAGGACTTTAGGCTCTGCGGCTCAGCAGGTGATGTCGTCGTCGGTGGG | 82.58% |
| Specimen No. NMR2A9-3 | AAATCTCAAACTTCACAGACAAAACAACTAAACACACGCACCTACCACGACAGGAGGACTTTAGGCTCTGCGGCTCAGCAGGTGATGTCGTCGTCGGTGGG | 80.32% |
| Specimen No. NMR2A9-4 | AAATCTCAAACTTCACAGACAAAACAACTAAACACACGCACCTACCACGACAGGAGGACTTTAGGCTCTGCGGCTCAGCAGGTGATGTCGTCGTCGGTGGG | 80.65% |

TABLE 3

Core nucleotide sequences of 4 indeterminate specimens of hepatitis C virus

| Specimen No. NMR2A9-1 | TGGCGCCCTGTATGGCGAGGAGGACTTTAGGCTCTGCGGCTCAGCAGGTGATGTCGTCGTCGGTGGG | 80.92% |
| Specimen No. NMR2A9-2 | TGGCGCCCTGTATGGCGAGGAGGACTTTAGGCTCTGCGGCTCAGCAGGTGATGTCGTCGTCGGTGGG | 81.24% |
| Specimen No. NMR2A9-3 | TGGCGCCCTGTATGGCGAGGAGGACTTTAGGCTCTGCGGCTCAGCAGGTGATGTCGTCGTCGGTGGG | 79.96% |
| Specimen No. NMR2A9-4 | TGGCGCCCTGTATGGCGAGGAGGACTTTAGGCTCTGCGGCTCAGCAGGTGATGTCGTCGTCGGTGGG | 82.83% |

The highest degree of homology.

TABLE 4

Comparison of indeterminate with reported genotypes within a 310-basepair core sequence of hepatitis C virus

| Specimen No. NMR2A9-2 | AAATCTCAAACTTCACAGACAAAACAACTAAACACACGCACCTACCACGACAGGAGGACTTTAGGCTCTGCGGCTCAGCAGGTGATGTCGTCGTCGGTGGG | 82.26% |
| Specimen No. NMR2A9-3 | AAATCTCAAACTTCACAGACAAAACAACTAAACACACGCACCTACCACGACAGGAGGACTTTAGGCTCTGCGGCTCAGCAGGTGATGTCGTCGTCGGTGGG | 82.26% |
| Specimen No. NMR2A9-4 | AAATCTCAAACTTCACAGACAAAACAACTAAACACACGCACCTACCACGACAGGAGGACTTTAGGCTCTGCGGCTCAGCAGGTGATGTCGTCGTCGGTGGG | 80.32% |

The highest degree of homology.
predominant number of samples analyzed were obtained from the blood donor population from the islands of Java and Bali. Analysis of a few positive isolates from the other islands of the Indonesian archipelago showed that most of these belonged to the HCV 1b genotype. Again, more detailed studies of a larger number of samples is required before any claims can be made regarding the genotype prevalence of HCV in the other Indonesian islands.

One other issue that needs to be addressed is the reason for the isolation and genotype identification of only 67 of the 102 HCV seropositive samples in the studies reported herein. It is reasoned that this is most likely due to any of the 3 following reasons or a combination thereof. It is possible that the individuals who were seropositive but failed to show the presence of the virus was due to the fact that the individuals were infected some time ago and had cleared their viremia prior to sample procurement. This is likely to be the most reasonable explanation for the data obtained. However, it is also possible that the failure to identify HCV in these samples was due to the limits of the sensitivity of the techniques used due to the unusual occurrence of variants that have sufficient differences in the regions of the primers and/or probes being used. Finally, it is possible that the EIA showed reactivity that was in fact false positive. Unfortunately, follow-up samples from these individuals were not available to distinguish among these possibilities. Finally, the significance of the results of the sequence alignments of the 4 indeterminate HCV samples needed to be addressed. Based on an arbitrary judgment, it appears that samples NMR2A9–1, NMR2A42–1, and NMR2A46–2 have the highest degree of homology with genotype 1c. Sample NMR2A75–5 appears to be the most divergent of the 4 indeterminate HCVs. Clearly, additional sequencing studies of these indeterminates and their alignment with corresponding determinate HCVs. Based on an arbitrary judgment, it appears that samples NMR2A9–1, NMR2A42–1, and NMR2A46–2 have the highest degree of homology with genotype 1c. Sample NMR2A75–5 appears to be the most divergent of the 4 indeterminate HCV isolates. Based on an arbitrary judgment, it appears that samples NMR2A9–1, NMR2A42–1, and NMR2A46–2 have the highest degree of homology with genotype 1c. Sample NMR2A75–5 appears to be the most divergent of the 4 indeterminate HCV isolates.

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