Antigenic Heterogeneity of the NS3 Proteins in Hepatitis C Virus Genotypes 1 and 6

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Key Words

Hepatitis C virus NS3 • Recombinant antigen • Heterogeneity • Enzyme immunoassay

Abstract

Objective: To investigate the effect of sequence variability between different genotypes of hepatitis C virus (HCV) on the antigenic properties of the NS3 proteins, and to evaluate the significance of the proteins in the serological diagnosis.

Methods: The recombinant expression plasmids of pET/NS3-1 and pET/NS3-6 were constructed with the NS3 region fragments from HCV genotypes 1 and 6, respectively. The antigenic reactivity of the recombinant NS3 proteins was determined. The NS3 nucleotide sequences were aligned with 6 HCV full-length strains representing different genotypes.

Results: The two recombinant NS3 proteins were generated and enzyme immunoassay tests were developed. The positive rates of anti-NS3 were 61.2 and 58.8% by using the NS3 antigens from HCV genotypes 1 and 6, respectively. This difference had no statistical significance ($p > 0.05$). However, it was interesting that 16 samples gave a discordant result for different genotype NS3 antigens. The two partial NS3 sequences shared approximately 63.9–79.9% identity with the different genotype strains. **Conclusion:** The primary structure of the HCV NS3 region is highly variable. Thus, sequence

variability has a profound effect on the antigenic properties of the NS3 regions, and the antigenic differences should be taken into account for the development of more effective diagnostic tests.

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Introduction

Hepatitis C virus (HCV) infection is a major public health problem with about 3% of the world population infected. Approximately 85% of HCV-infected patients develop chronic infection, and about 20% of chronic cases will progress into cirrhosis and/or hepatocellular carcinoma [1–5]. To date, there is no vaccine available against HCV, and the only available therapy, interferon- α on its own or in combination with ribavirin, is effective in only a minority of patients and carries the risk of serious side effects [6–12]. Presently, the screening of blood and blood products for a specific antibody to HCV is an effective means for preventing the transmission of HCV, especially in high-risk groups such as intravenous drug users, hemodialysis patients and organ grafters [13, 14].

Today, third-generation enzyme immunoassays (EIAs) are widely used and have higher degrees of sensitivity and specificity than second-generation assays. A

sharp drop in post-transfusional hepatitis C was observed when screening assays for HCV antibodies were applied to blood donors [15]. However, patients of post-transfusion and sporadic hepatitis C infection were still reported. Thus, these assays are prone to false-negative results, which demonstrates that the current EIA is not yet perfect [15–17].

The most widely used serologic diagnostic tests for HCV infection are currently based on the detection of antibodies to viral antigens using a combination of various HCV proteins molecularly expressed *in vitro* based on the American prototype HCV isolate 1a [18]. This is problematic when applying the test in a region where other genotypes of HCV are dominant [19]. Evidence was accumulating that non-HCV type 1 isolates showed less reactivity in these assays to some recombinant non-structural proteins [18]. Kanistanon et al. [19] have previously reported that commercial assays could not detect HCV antibodies in some Thai patients who were infected with HCV. They found that the immunoreactivities to NS4 antigens in the commercial HCV assay were significantly less frequently detected in samples from patients infected with genotypes 1b, 3, and 6 or the unclassified HCV genotypes, compared to those of genotype 1a.

It is thought that the variability in serological reactivities among HCV-infected patients may result from antigenic heterogeneity of different genotypes. Serodiagnosis of HCV is based on the use of recombinant proteins and/or peptides corresponding to various regions of core: NS3, NS4, and NS5. On that basis, the localization of the immunodominant epitopes of HCV proteins and, more particularly, of NS3 is important for the construction and development of sensitive HCV immunoassays [16]. The NS3 protein, which showed strong immunoreactivities to HCV-infected blood samples and the anti-NS3 antibody, appeared early in the course of infection and is likely to play a pivotal role in the serological assays of HCV [19]. The antigenic compositions of many HCV proteins have been studied by using recombinant proteins and synthetic peptides [16, 20]. However, the influence of the NS3 genomic variability on the serological diagnosis of HCV by EIA remains poorly defined, and, therefore, few attempts have been made to improve the diagnostic value of this antigen. In the present study, recombinant NS3 proteins were expressed using genes cloned from HCV genotypes 1 and 6, respectively. We compared the serological reactivity of the two proteins and assessed their antigenic heterogeneity.

Materials and Methods

Vectors and Strains

Recombination plasmid pGEX-4T-2/NS3 containing the HCV NS3 gene segments from HCV genotypes 1 and 6 were obtained from the Department of Microbiology and Immunology, Southeast University, Nanjing, China. Expression vector pET-28a-c(+), containing T7 promoter and an N-terminal 6× His tag, multiple cloning sites and a anti-kanamycin sulfate coding sequence, was purchased from Novagen Co., USA. *Escherichia coli* BL21 (DE3) was conserved in our laboratory and used as expression host.

Serum Specimens

HCV-positive sera ($n = 85$) were obtained from different hospitals in China and were seropositive for IgG antibody against HCV, while seronegative for infectious markers of both hepatitis A and B. Anti-HCV-negative serum specimens ($n = 100$) were obtained from healthy blood donors. The anti-HCV status was determined by anti-HCV EIA kit (Huamei Biological Engineering Inc., China). These specimens ($n = 39$) from an anti-HCV-negative panel were kindly provided by the Department of Microbiology and Immunology, Southeast University. The HCV genotype was determined by a type-specific primer assay based on 5' non-coding region [21]. All human sera were stored at -70° prior to the analysis.

Construction and Identification of Recombinant Plasmids

pET-28a-c(+)/NS3

The two recombinant plasmids were constructed using pGEX-4T-2/NS3, which contained NS3 partial sequences from HCV genotypes 1 and 6, respectively. The NS3 fragments were digested from the pGEX-4T-2/NS3 vector by using *Bam*HI and *Xho*I restriction enzymes (Boehringer Mannheim, Germany) at 37° overnight, and then subcloned into the same sites in pET-28a-c(+) vectors with T4 DNA ligase (Pharmacia Biotech, USA) at 16° overnight. The two constructs were named pET/NS3-1 and pET/NS3-6, representing NS3 regions derived from HCV genotypes 1 and 6, respectively. The resulting plasmids were both transformed into *E. coli*-competent BL21 (DE3) cells using kanamycin resistance for selection. Recombinant plasmids were recovered from transformants by using the Wizard Miniprep DNA Purification System (Promega). The presence of insert was identified by restriction enzymes and PCR using 5'-TAATACGACTCACTATAGGG-3' as the forward primer and 5'-TGCTAGTTATTGCTCAGCGG-3' as the reverse primer, which both derived from regions flanking the multiple cloning site of pET-28a-c(+). The PCR condition was initially denatured at 94° for 30 s; each cycle of amplification consisted of 94° for 45 s, annealing at 51° for 30 s and polymerization at 72° for 90 s, and further polymerization for 10 min after 25 PCR cycles. The primary structure of the inserts (804 bp) was finally confirmed by DNA sequencing with the automatic sequencer (ABI, USA) (data not shown).

Expression and Purification of Recombinant NS3 Proteins

The *E. coli* BL21 cells transformed with the recombinant plasmids were incubated overnight at 37° in Luria broth medium containing 50 μ g/ml kanamycin. The overnight culture was diluted 20 times with fresh Luria broth medium containing the same concentration of kanamycin and grown at 37° for 3–4 h until an optical density value of 0.4–0.6 at 600 nm was reached. The gene expression was induced by adding isopropyl- β -D-thiogalactoside (IPTG; Sigma) into the culture to a final concentration of

Fig. 1. Expression and purification of recombinant HCV NS3 proteins derived from genotype 1 (a) and genotype 6 (b) in *E. coli* BL21. Samples were analyzed on standard reductive SDS-PAGE followed by Coomassie brilliant blue staining. Lanes: M = protein molecular weight marker; 1 = induced pET as negative control; 2 = *E. coli* BL21 pET/NS3 uninduced; 3 = whole-cell sample after induction with IPTG; 4 = purified products with Ni-NTA Superflow.

1 mmol/l. After 4 h of incubation at 37° with constant shaking, the cells were pelleted by centrifugation at 6,000 g for 15 min at 4° and then treated by lysozyme (Sigma). Cell lysates were subsequently prepared for protein purification. The induced polyhistidine-tagged NS3 proteins were purified under denaturing conditions in the presence of 8 mol/l urea using a Ni-NTA agarose affinity chromatography system (Qiagen) with an elution step. This procedure was carried out according to the protocol of the manufacturer. The urea was removed from the protein preparation after dialysis against phosphate-buffered saline (PBS).

Enzyme Immunoassay

All the serum samples were tested at a 1:100 dilution employing EIA based on coating of the solid phase with recombinant HCV NS3 proteins derived from HCV genotypes 1 and 6, respectively. To optimize plate coating, purified proteins were titrated to determine an optimal concentration (data not shown) at which a pool of 5 positive serum specimens from patients infected with HCV and negative controls were maximally discriminated. The two NS3 proteins were then adsorbed onto microtiter wells in PBS (pH 7.4), overnight at 4°, respectively. The wells were washed with PBS-Tween buffer (0.1 mol/l PBS containing 0.05% Tween-20) 3 times. Serum specimens were diluted in blocking solution and incubated in the microtiter wells with the preadsorbed recombinant protein for 1 h at 37°. After the microtiter wells were washed, goat anti-human immunoglobulin G (IgG)-conjugated to horseradish peroxidase (Pierce) diluted 1:4,000 in blocking solution was added to the wells and incubated for 1 h at 37°. The microtiter wells were washed again 5 times and then subjected to color development by adding tetramethylbenzidine substrate. The plates were incubated for 10 min in the dark, and the reaction was stopped with 50 µl of 2 mol/l sulfuric acid, and the absorbance (A) of the reaction was measured at 450 nm. The results were evaluated by calculating an S/C value, where S is the A value of detected sera, and C is the value statistically determined as the mean A value of negative control serum samples. An S/C value >2.1 was considered positive.

Western Blotting

The two NS3 proteins were denatured by boiling in Laemmli sample buffer for 5 min and electrophoresed in a single-well 10% polyacrylamide gel. Electrophoretic transfer of the proteins to a nitrocellulose membrane was carried out at 126 mA for 1 h at 4°. The membrane was blocked for 30 min with 5% skim milk in PBS

and then cut into strips prior to overnight incubation at 4° with different anti-HCV-positive serum specimens. After washing the membrane 3 times for 10 min each in PBS-Tween, a goat anti-human IgG-conjugated with horseradish peroxidase secondary antibody (Pierce) was added at a dilution of 1:1,000 in 5% skim milk-PBS. After 2 h, the blots were washed 3 times with PBS-Tween for 10 min each, and then detection was performed by using enhanced chemiluminescence Western blotting detection reagent (Amersham Biosciences) according to the manufacturer's protocol.

Computer-Assisted Sequence Analysis

The HCV NS3 partial nucleotide sequences from different genotypes were aligned with 6 HCV full-length strains representing different genotypes from GenBank. The alignments were analyzed using a multiple-alignment algorithm (Clustal method) in the MegAlign program from the Lasergene software package (DNASTAR Inc., Madison, Wisc., USA). Abbreviations used for the HCV full-length strains and the GenBank accession numbers in the analyses are as follows: AB049088 (genotype 1b), AF177036 (genotype 2a), D17763 (genotype 3a), Y11604 (genotype 4a), Y13184 (genotype 5a), and Y12083 (genotype 6a).

Statistical Analysis

χ^2 test was used to analyze the data. $p < 0.05$ was considered statistically significant.

Results

Expression of pET-28a-c(+)/NS3 in *E. coli* and Protein Purification

The recombinant NS3 proteins used in the study were produced in *E. coli* BL21 cells. The expression of polyhistidine-tagged NS3 was induced by IPTG and purified by affinity chromatography. The purified fusion NS3 proteins were both analyzed by electrophoresis in a 10% polyacrylamide gel (fig. 1). The results showed that the clearly identifiable bands were similar to that predicted, and the expected sizes of the NS3 proteins were about 31.5 kDa (genotype 1) and 31.2 kDa (genotype 6), respectively.

Fig. 2. The different antigenic reactivity to 16 sera by NS3-1 (black) and NS3-6 (white). The anti-NS3 detection by EIA is described in Materials and Methods. The absorbance (A) of the anti-NS3 reaction was measured at 450 nm. Significant changes ($A_{450\text{ nm}}$) were observed between NS3-1 and NS3-6.

Fig. 3. Western blotting analyses of recombinant HCV NS3 proteins derived from genotypes 1 and 6. The NS3 proteins were separated by SDS-10% PAGE and then transferred to nitrocellulose membranes. After being immunoblotted with different anti-HCV-positive serum specimens, secondary antibody conjugated with horseradish peroxidase was added, and the results were detected with an enhanced chemiluminescence system. Lanes: 1 = pET control with anti-HCV-positive specimen; 2 = uninduced cell lysate with anti-HCV-positive specimen; 3 = purified NS3-1 with anti-HCV-negative specimen; 4 = negative reaction of purified NS3-1 with anti-HCV-positive specimen; 5 = positive reaction (arrow) of purified NS3-1 with anti-HCV-positive specimen; 6 = purified NS3-6 with anti-HCV-negative specimen; 7 = negative reaction of purified NS3-6 with anti-HCV-positive specimen; 8 = positive reaction (arrow) of purified NS3-6 with anti-HCV-positive specimen.

Immunoreactivity of Recombinant Proteins

The two NS3 recombinant proteins from different HCV genotypes were generated and EIA tests were developed. 100 serum samples from healthy blood donors and 39 specimens from a negative panel, which were shown to be anti-HCV-negative, were examined with the two NS3 antigens. These samples were with both anti-NS3-negative. Meanwhile, 85 anti-HCV-positive serum samples were tested for anti-NS3 activity. The positive rates of anti-NS3 were 61.2% (52/85) by using the NS3 antigen from HCV genotype 1 and 58.8% (50/85) by using the antigen from genotype 6, respectively. But this

difference between the two positive rates of anti-NS3 did not reach statistical significance ($\chi^2 = 0.06$). However, it was interesting that 16 samples gave a discordant result. Thus, 7 specimens detected with the NS3 antigen from genotype 1 were negative, but positive when detected with the antigen from genotype 6. In contrast, 9 specimens were negative with the HCV NS3 antigen from genotype 6, but were positive with the antigen from genotype 1 (table 1; fig. 2). To further confirm the discordant result, a Western blotting assay was performed for the 16 discordant samples. Meanwhile, the pET-28a-c(+) blank control and uninduced cell lysate specimens were tested with an anti-HCV-positive specimen as well as an anti-HCV-negative specimen in parallel. The results are shown in figure 3. None of the anti-HCV-negative samples reacted with any of the two recombinant NS3 proteins of either genotype. Of the 16 discordant samples tested, only 9 reacted with genotype 1 NS3 and not with genotype 6 NS3. Another 7 specimens, which were not immunoreactive with the recombinant NS3 protein from genotype 1, were immunoreactive with the recombinant NS3 protein from genotype 6. In addition, it was shown that there were negative reactions of pET-28a-c(+) blank control as well as uninduced cell lysate with anti-HCV-positive specimens. In order to further compare the immunoreactivity of the NS3 proteins from genotypes 1 and 6, the HCV genotype was identified by a type-specific primer assay according to the sequence of 5' non-coding region to the 16 discordant samples. The results are presented in table 2.

Analysis of NS3 Gene Nucleotide Sequences

The two partial HCV NS3 sequences were both sequenced and belonged to genotype 1 and genotype 6, respectively. Their sequences shared approximately 68.4–79.9% (genotype 1) and 63.9–70.0% (genotype 6) identity

Table 1. Comparison of anti-NS3 detection among 16 discordant samples

Sample No.	NS3-1			NS3-6		
	A _{450 nm} ^a	S/C ^b	result ^c	A _{450 nm} ^a	S/C ^b	result ^c
L22	1.208	20.1	+	0.065	1.1	–
L73	1.460	24.3	+	0.063	1.1	–
L78	1.047	17.5	+	0.069	1.2	–
L87	0.895	14.9	+	0.070	1.2	–
H4	1.739	29.0	+	0.079	1.3	–
H31	1.375	22.9	+	0.065	1.1	–
H61	0.928	15.5	+	0.049	0.8	–
H24	1.563	26.1	+	0.068	1.1	–
C17	0.809	13.5	+	0.041	0.7	–
L27	0.057	1.0	–	0.908	15.1	+
L63	0.038	0.6	–	0.940	15.7	+
L81	0.036	0.6	–	1.233	20.6	+
L83	0.057	1.0	–	0.875	14.6	+
H1	0.062	1.0	–	1.031	17.2	+
C2	0.045	0.8	–	0.986	16.4	+
C14	0.056	0.9	–	1.135	18.9	+

The anti-NS3 detection by EIA is described in Materials and Methods.

^a The absorbance (A) of the anti-NS3 detection was measured at 450 nm. ^b S is the A value of detected sera, and C is the value statistically determined as the mean A value of negative control serum samples. In the text, the mean A value of C calculated was 0.06. ^c The S/C values under and above 2.1 are indicated as – and +, respectively.

Table 2. Immunoreactivity comparison of NS3 proteins derived from different genotypes with serum samples from various HCV genotypes

Sample genotype	Immunoreactivity with NS3 proteins from different genotypes	
	NS3 (genotype 1)	NS3 (genotype 6)
1 (n = 13)	9	4
2 (n = 1)	0	1
Unclassified (n = 2)	0	2
Total (n = 16)	9	7

with the corresponding region of HCV-representative different genotype strains at the nucleotide level. The homology of the HCV NS3 nucleotide sequences was 69.9% between HCV genotypes 1 and 6, and the deduced amino acid identities were 83.2% (fig. 4, 5). Many of these changes were third position substitutions that did not result in differences at the amino acid level. Thus, the amino acid

differences between the NS3 sequences from genotype 1 and genotype 6 were not significantly different from those of the nucleotide level. The NS3 comprised both 804 nucleotides with a coding capacity of 268 amino acids.

Discussion

The HCV genome, a positive single-strand RNA of about 9,500 nucleotides, is composed of a structural region and a non-structural region subdivided into six parts (NS2, NS3, NS4A/B, NS5A/B) [22, 23]. The viral genome is characterized by a high genetic heterogeneity. Four levels of genetic variability have been described: types, subtypes, isolates and quasispecies. The nucleotide sequence data analyses on the different isolates have demonstrated at least six major genotypes, and an increasing number of subtypes have been reported since its discovery [24]. Meanwhile, in infected individuals, HCV exists as a variable complex population of related genetic variants known as quasispecies [22, 25]. HCV genotypes differ in their complete genome sequences by 30% or more at the nucleotide level. Each of these virus types is comprised of several subtypes that differ from each other by about 20%. In turn, the virus sequences comprising each virus subtype differ by up to 10% [24]. The HCV genotype distribution can vary significantly in different geographical areas of different countries, or even in the same country, as has been observed for example in China [26–30]. Genotype 1 is widely distributed in China and constitutes a significant proportion of HCV isolates, however, many non-1 genotypes have been identified in China. On the other hand, the observed distribution of HCV genotypes could vary depending on which group of individuals was studied, and the genotype distribution has been found to be different in different patients and at different stages of HCV infection [31–33]. Overall, many genotypes are widely distributed in the world, and a significant heterogeneity of HCV has been reported among strains from different geographical areas [24, 26]. The genetic diversity of HCV, that may result in some differences in antibody reactivity, is of potential concern for the sensitivity of diagnostic assays based on antigens derived from different genotypes and subtypes. Thus, the current commercial HCV tests are based on antigens from HCV type 1 only. These tests have proved highly successful in improving the safety of donated blood and in providing a wealth of new knowledge about events following HCV infection in patients worldwide. However, commercially available HCV serological tests based on

Fig. 4. Alignment of the deduced amino acid sequences of HCV NS3 recombinant proteins derived from genotypes 1 and 6. The numbers of amino acid positions are on the left, and identical amino acid residues at each position are indicated by dots.

1	VDFIPVEGDPTTMRSPVFTDNSSPPAVPES	NS3 (genotype 1)
	L..V.C.AMDA.T...T.....T.....QA	NS3 (genotype 6)
31	FQVAHLHAPTGSGBKSTKVPAAYAAQGYKVL	NS3 (genotype 1)
	Y..GY.....V..S.....	NS3 (genotype 6)
61	VLNPSVAATLGFGAYMSKAHGIDPNIRTGV	NS3 (genotype 1)
	S..S.L.R.....	NS3 (genotype 6)
91	RTITTGSPITYSTYKFLADGGCSGGAYDI	NS3 (genotype 1)
	A.....V	NS3 (genotype 6)
121	TICDECHSTDATSILGIGTVLDQAETAGAR	NS3 (genotype 1)
	P.TV.....C.	NS3 (genotype 6)
151	LVVLATATPPGSVTVPHPNIEEVALSTTGE	NS3 (genotype 1)
	.T.....Q.T..PL...	NS3 (genotype 6)
181	IPFYGKAIPLEVIKGRHLIFCHSKKKCDE	NS3 (genotype 1)
	V.....Y.....	NS3 (genotype 6)
211	LAAKLVALGINAVAYYRGLDVSVIPTSGDV	NS3 (genotype 1)
	...Q.RT..L....F...V.....	NS3 (genotype 6)
241	VVVATDALMTGYTGDFDSVIDYNTCVT.	NS3 (genotype 1)
	..C.....C.VA...	NS3 (genotype 6)

Fig. 5. Comparison of nucleotide identities and genetic distances between HCV NS3 partial gene sequences and 6 HCV different full-length gene sequences.

	1	2	3	4	5	6	7	8		
1		69.4	67.5	68.3	67.7	67.3	72.5	66.2	1	AF177036 2a
2	40.7		69.2	71.6	70.7	69.9	79.9	66.3	2	AB049088 1b
3	45.5	41.3		69.4	68.8	68.5	68.4	63.9	3	D17763 3a
4	43.2	36.7	42.2		69.7	69.2	75.0	67.8	4	Y11604 4a
5	44.2	38.8	41.7	41.1		68.8	72.4	67.0	5	Y13184 5a
6	45.5	40.4	43.5	42.0	42.6		71.3	70.0	6	Y12083 6a
7	35.1	23.8	41.5	30.6	35.9	36.6		69.9	7	NS3 (1)
8	45.4	44.1	46.1	41.3	43.8	38.5	40.3		8	NS3 (6)
	1	2	3	4	5	6	7	8		

proteins or peptides derived solely from HCV genotype 1 may be less effective for the detection of antibody against different HCV genotypes with equal efficiencies [18].

The NS3 protein is one of most important antigenic targets of HCV diagnostic kits. However, the variability of HCV is distributed throughout the genome with sequences coding for the non-structural proteins such as NS3 showing comparable degrees of variability to the mean values over the complete genome [22, 24]. In the present study, the NS3 genes of HCV genotypes 1 and 6 were compared with different complete genome isolates representing different genotypes by the unweighted pair-group method using the computer software package. The level of nucleotide and deduced amino acid sequence analysis showed the NS3 similarities were 69.9 and 83.2% between HCV genotypes 1 and 6, respectively. Compared to differ-

ent full-length genome strains, the NS3 sequences shared approximately 68.4–79.9% (genotype 1) and 63.9–70.0% (genotype 6) sequence identity at the nucleotide level. The comparisons of the sequence identities indicated that the NS3 sequences were significantly divergent among different genotype isolates. Nucleotide sequence variation may result in changes in the amino acid sequences of expressed NS3 recombinant proteins and thus in possible variations in antigenic determinants. It is therefore conceivable that such a significant nucleotide sequence variability may significantly affect the antigenic properties of this region.

There is now increasing evidence that HCV type 1-based assays are not detecting other HCV genotypes with equal efficacy. A significantly lower serologic reactivity of current HCV type 1-based EIA with samples from donors with non-HCV type 1 has been reported [16,

18], particularly to NS4 antigens [18, 20]. An analysis of different amino acid sequences from different HCV genotypes showed that the primary structure of region NS4 was considerably variable and that the range of sequence similarity was between 32.4 and 98.6% [20]. Such a significant heterogeneity of the primary structure of the HCV NS4 protein has a substantial effect on its antigenic properties, which has resulted in the differences in serologic reactivity [20]. Similar previous studies have demonstrated that the primary structure of the NS5 antigenic region was also very variable and that such a significant sequence variability significantly affected the antigenic properties of this region. For example, Dou et al. [16] demonstrated that NS5 protein from genotype 2b had a better immunoreactivity with genotype 2 serum specimens than with other genotypes.

In this study, we have established an antibody assay for the NS3 antigens (anti-NS3) using 268 amino acids of the NS3 proteins expressed in *E. coli* from different genotypes. The anti-NS3 assay was highly specific, and 100 serum samples from healthy blood donors and 39 specimens from a negative panel were all anti-NS3-negative. The positive rates of anti-NS3 among 85 anti-HCV-positive serum specimens by using the NS3 antigen from genotypes 1 and 6 were 61.2 and 58.8%, respectively, but this difference did not reach statistical significance. However, a significant difference still existed in the reactivity of HCV genotypes 1 and 6 to the NS3 antigen. Thus, the recombinant NS3 antigen from genotype 1 could react with 9 specimens that did not react to the antigen from genotype 6 in the assay. Conversely, 7 specimens were negative with the HCV NS3 antigen from genotype 1 but were positive with the antigen from genotype 6. The type-specific primer assay was used for HCV genotyping and the 16 discordant samples were classified into genotype 1 (13), 2 (1) and unclassified (2). It was interesting that the NS3 protein (genotype 6) could react with 4 samples of genotype 1, which did not react with the NS3 protein from genotype 1. The results demonstrated that the NS3 proteins did not always have strong and strict immunoreactivity with the corresponding HCV genotype serum specimens. Similarly, a previous study demonstrated that the NS5A major antigenic region did not have a strict genotype-specific antigenic reactivity [16]. The significant variation in immunoreactivity between different genotype NS3 proteins may be explained in the study. This antigen difference in reactivity is most likely related to the sequence differences between the genotypes. In the study reported here, the comparison of the nucleotide identities demonstrated that the NS3 sequence of geno-

type 1 was significantly divergent from that of genotype 6 (69.9%), as well as six representative different genotype isolates (63.9–79.9%). Since significant heterogeneity was found for the primary structure of different genotype NS3 sequences, it resulted in several cases of differences in antibody reactivity and had a substantial effect on the antigenic properties of the NS3 proteins. In addition, Utama et al. [33] considered that HCV isolates may exist between different genotypes and that a virus recombination event could naturally occur during virus replication. The HCV recombination is a cause of genetic diversity of the virus, which may also have an important implication for HCV antibody detection. Thus, the genetic heterogeneity of HCV may result in false-negative results in assays for detecting HCV antibodies because sequences mismatch between the genotype 1-based assays currently used and the serum for detecting different genotypes. Even though the third-generation EIA are currently used, these differences in reactivity to HCV genotypes still remain [16].

In conclusion, the data obtained in the present study revealed that the NS3 region exhibited significant genetic heterogeneity, which had a prominent effect on the antigenic properties of the NS3 proteins. Therefore, antibodies from patients infected with different HCV genotypes may not cross-react with NS3 antigens from different genotypes due to the low homology to heterologous genotypes. This observation is important and should be taken into consideration in the further development of diagnostic assays for the detection of anti-HCV activity. On the other hand, the HCV genotype distribution can vary significantly in different geographical areas of the world, and the genotypes of patients infected with HCV may be unknown for screening populations. These findings, along with the observation that previous similar studies showed the different serological reactivity of different region proteins from different genotypes in detecting infection with the different genotypes of HCV, strongly suggest that antigenic targets should be carefully selected from different sequence variants based on breadth and strength of immunoreactivity when selecting antigenic targets for the development of highly sensitive serological diagnostic tests [16, 18, 19].

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