ASIAN PACIFIC JOURNAL OF ALLERGY AND IMMUNOLOGY (1997) 15: 133-140

The Immunoreactivity Profile of Different HCV Genotypes on Immunoblot Assay and Its Implications in the Development of Diagnostic Assays

Duangjit Kanistanon, Montana Neelamek, Tararaj Dharakul and Sirirurg Songsivilai

SUMMARY **The immunoreactivity profiles of plasma samples obtained from patients infected with different hepatitis C virus (HCV) genotypes were studied using immunoblot assay containing multiple HCV antigens. The immunoblot assay was found to be positive in 81.5% of 195 blood donors who had anti-HCV antibodies as detected by second generation enzyme immunoassays. The samples reacted preferentially with the viral core, NS3-1 and NS5 antigens, and these reactivities were not influenced by HCV genotype. However, the reactivities with NS3-2 and NS4 antigens varied depending on HCV genotypes. The samples from patients infected with HCV genotype 1 reacted well with NS3-2 and NS4 antigens whereas those with other genotypes did not. In addition, samples with the unclassified HCV genotype reacted poorly with all antigens, except NS3-1. This study demonstrates the importance of the core, NS3-1 and NS5 antigens in the detection of antibodies against HCV, especially in areas where more than one genotypes of HCV are present. It also demonstrates that there is a need for further improvement of the currently used assays as new HCV genotypes are recently discovered.**

Hepatitis C virus (HCV) causes transfusion associated hepatitis in patients receiving HCV contaminated blood or blood products. The consequences of HCV infection include chronic hepatitis which occurs in more than half of the infected individuals and leads to the development of cirrhosis and hepatocellular carcinoma in some cases. Genetic heterogeneity of this virus was demonstrated from the analysis of the nucleotide sequences of HCV isolated around the world and HCV was classified into at least 6 major genotypes (1-6) with a series of several subtypes, designated as a, b, c, etc.[1, 2] Genotypes 1, 2 and 3 are distributed in several regions around the world (America, Europe and Asia). Genotype 4 is found restrictedly in Africa continent whereas genotypes 5 and 6 are predominant in the Middle east and Southeast Asia regions, respectively.

The current method for diagnosis of HCV infection is based on the detection of antibodies against HCV in serum or plasma using enzyme immunoassays (EIA). The first generation EIA included only the C100-3 antigen of the nonstructural 4 (NS4) protein of HCV and further studies found that this test gave false negative results in a large number of samples.[3,4] The second and third generation EIA were later developed by including more antigens from both structural (core protein) and nonstructural (NS3, NS4 and NS5) proteins in the assays, greatly improving their sensitivity.[5] However, some samples still gave false negative results in the detection of anti-HCV antibodies.[6,7] The most reasonable cause for this

From the Department of Immunology, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok 10700, Thailand
Correspondence: Sirirurg Songsivilai

false negative result was the genetic variation of the virus since the assays were developed based on the available information on HCV genotype 1. Therefore, antibodies elicited against the infecting viral genotype may have limited ability to crossreact to antigens used in the assays. In addition to HCV EIA, HCV immunoblot assays were also developed using both the structural and nonstructural antigens, and provided the confirmatory tests for the presence of anti-HCV antibodies. The reactivities of sera from patients infected with each HCV genotype to each antigen could be analysed in the immunoblot. Several studies showed that samples containing other genotypes such as 2a and 2b gave lower reactivities in these assays compared to the samples of genotype 1.[5,8,9] At present, no gold standard method for diagnosis of HCV viremia is available. HCV RNA assay may not be able to detect low levels of HCV viremia and HCV EIA also misses some samples of HCV viremia detected by RT-PCR.

The distribution pattern of HCV genotypes found in Thailand is significantly different from that in some other regions of the world. HCV genotype 3a is the most common genotype in this country, followed by genotypes 1b, 6 group variants, 1a and 3b.[10] Since the prevalence of HCV infection in Thai blood donors ranges from 1.5% to as high as 5% depending on the region where the samples are obtained,[11] assays that could efficiently detect the presence of anti-HCV antibodies in blood donors would be very valuable in the prevention of HCV transmission. However, the effects of the common HCV genotypes in this country, especially genotypes 3 and 6 group variants, on the immunoreactivities to viral antigens has not been documented. We therefore analyzed the pattern of the immunoreactivities in samples with different HCV genotypes with a panel of HCV antigens. The results from this study could be useful as the basis for the development of diagnostic assays in the future.

MATERIALS AND METHODS

Specimens

A total of 195 anti-HCV-positive plasma samples were collected from healthy blood donors attending Blood Transfusion Centers at Siriraj Hospital, Bangkok, and Khonkaen Regional Hospital, Khonkaen, Thailand. Self-exclusion criteria had been applied to these donors prior to blood donation, and none had a previous history of hepatitis. Antibodies to HCV were assayed by second generation enzyme immunoassays (EIA, Organon, United Biomedical, USA; Murex, England and Diagnostic Biotechnology, Singapore). Seropositivity was confirmed by using EIA from other manufactures which differed from that used in the initial screening. In addition, samples from Khonkaen were also tested for anti-HCV antibodies using particle agglutination assay (Serodia, Japan) and were confirmed by EIA (Organon). Plasma were aliquoted into 1.5 ml vials and immediately stored at -70°C.

HCV immunoblot assay

The reactivities of immunoblot assay were determined by using HCV BLOT 3.0 assay (Genelabs Diagnostics, Singapore) which consisted of core, NS3-1, NS3-2, NS4 and NS5 antigens. The procedures and interpretation of the assay were performed according to the manufacturer's instruction. The reactivities of antibodies in the plasma samples to individual antigens blotted onto the nitrocellulose membrane were recorded. Positive results in the immunoblot assay were documented when antibodies in the sample reacted to at least 2 antigens. The results were interpreted as indeterminate if antibodies reacted to only one of the antigens. Negative results meant that the plasma could not react with any of the antigens.

HCV RNA assay

HCV RNA was detected in plasma samples using a reverse transcription-nested polymerase chain reaction (RT-PCR) amplification essentially as previously described.[10] In brief, RNA was extracted from plasma using a modified guanidium thiocyanate phenol chloroform extraction method. RNA was converted into cDNA by reverse transcriptase enzyme at 42°C for 30 minutes, and then subjected to first round of PCR amplification using primers covering the 5'-noncoding region (5'-NCR). The PCR profile consisted of 35 cycles of 94°C 1 minute, 40°C 1 minute and 72°C 2 minutes. The final extension step was carried out at 72°C for 10 minutes. One-tenth of the primary PCR-amplified product was taken to second round amplification using the same thermal cycling profile. The final PCR-amplified product was analyzed by agarose gel electrophoresis and visualized under

UV light after staining with ethidium bromide.

HCV genotype determination

HCV genotypes in samples containing HCV RNA were determined by 2 reverse hybridization assays as previously described.[10] Briefly, genotype/subtype-specific oligonucleotide probes immobilized on nitrocellulose membranes were allowed to react with biotin-labeled PCR product amplified from either HCV-positive or -negative specimens. The PCR product of a certain genotype/subtype would hybridize only with the corresponding genotype/subtype-specific probes. The binding was visualized by streptavidin-mediated enzymatic reaction. One assay was based on the nucleotide sequence within 5'-NCR and the other on a nucleotide sequence within the core gene. The 5'-NCR-based genotyping assay consisted of oligonucleotide probes specific to genotype 1, 1a, 1b/6 group variants, 3a and 3b. A universal probe specific for every genotype known to date, as well as positive and negative control probes were also included in the assay. Since the 5'-NCR-based genotyping assay could not differentiate between genotype 1b and 6 group variants, the core-based genotyping assay was applied to discriminate these two genotypes. This assay contained two probes, one could bind to genotype 1b whereas the other could bind to genotype 6 group variants. Positive and negative control probes were also included in this assay.

RESULTS

HCV immunoblot assay

Immunoblot assay was performed on 195 anti-HCV-positive samples and was found to be positive in 81.5%, indeterminate in 5.7% and negative in 12.8% of the samples (Table 1). Almost all of the samples in the immunoblot-positive group reacted to the core antigen, compared with only 72% in the immunoblot-indeterminate group (Fig. 1). Reactivities with NS3-1, NS3-2, NS4 and NS5 antigens of the immunoblot-positive group were found in 88.7%, 51.6%, 45.3% and 86.6% of the samples, respectively. However, only 18.2% and 9.1% of the immunoblot-indeterminate samples reacted with NS3-1 and NS4 antigens, respectively, and none reacted with NS3-2 and NS5 antigens (data not shown). An example of the results is shown in Fig. 2.

Table 1 Results of the HCV RNA detection and immunoblot assay

HCV RNA	Number of samples with immunoblot result as			Total
	Positive	Indeterminate	Negative	
Positive	136	1[a]	-	137
Negative	23	10	25	58
Total	159 (81.5%)	11 (5.7%)	25 (12.8%)	195

[a] A sample of genotype 1a which reacted to the core antigen only

HCV RNA assay

HCV RNA was detected in 70.3% of anti-HCV-positive plasma samples. HCV RNA was found in 85.5% of the immunoblot-positive samples, and also in one (9.1%) sample of the immunoblot-indeterminate group (Table 1). The viral RNA could not be detected in any of the samples with immunoblot-negative results.

Genotyping of HCV

Reverse hybridization assays were performed on samples containing HCV RNA and the results are shown in Table 2. In addition, one sample of the immunoblot-indeterminate group contained HCV genotype 1a.

Immunoblot assay reactivities in different HCV genotypes

The reactivity profiles of immunoblot assay of each HCV genotype are shown in Table 2 and Fig. 3. The samples of every genotype reacted well with core, NS3-1 and NS5 antigens, with average positive rates of 97%, 90% and 85%, respectively. The reactivities to NS3-2 and NS4 antigens were dependent on the HCV genotype, and antibodies were frequently detected in samples of genotype 1, but poorly detected in the samples of the other genotypes.

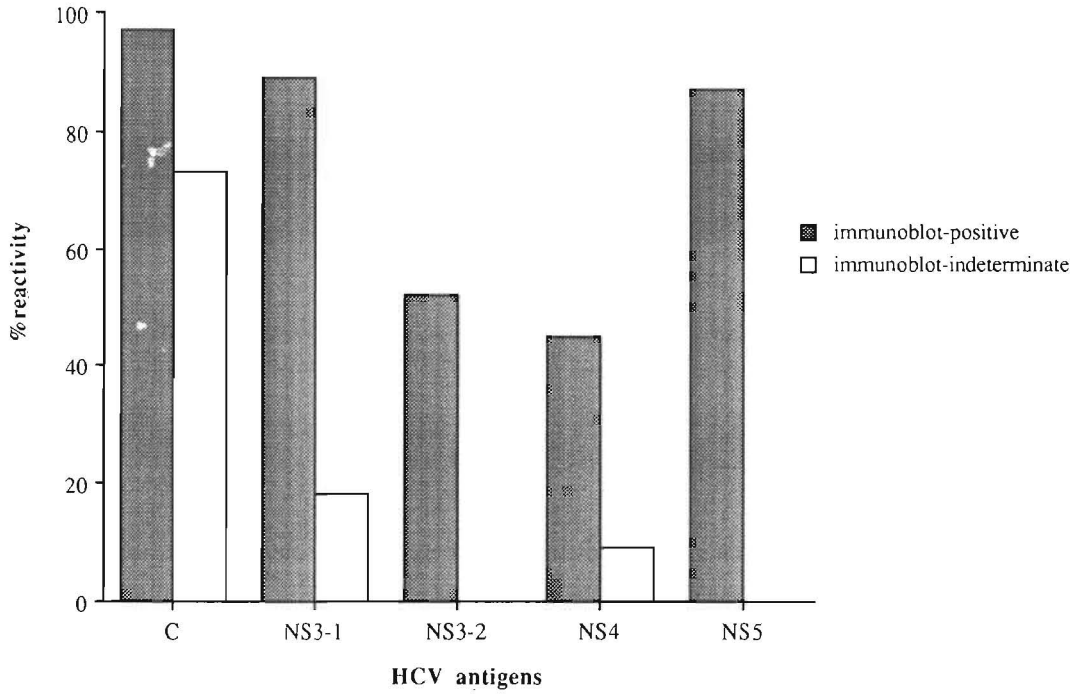

Fig. 1. Percentage of the immunoblot-positive and immunoblot-indeterminate samples which reacted with individual antigens in the immunoblot assay

Table 2 Genotyping distribution and the immunoreactivity with individual HCV antigens in the immunoblot-positive plasma samples containing different HCV genotypes

HCV genotype	n	% of samples reactive with individual antigens				
		Core	NS3-1	NS3-2	NS4	NS5
1a	15	93	100	93	86	93
1b	25	96	96	**60**	92	84
3a	56	100	82	**42**	**28**	87
3b	5	100	80	**60**	**25**	78
6 group variants	28	100	92	**67**	**25**	78
Unclassified	7	71	100	**28**	**42**	71
HCV RNA positive	136	97	90	**57**	**48**	85
HCV RNA negative	23	95	82	**21**	**30**	95
otal	159	97	89	**52**	**45**	87

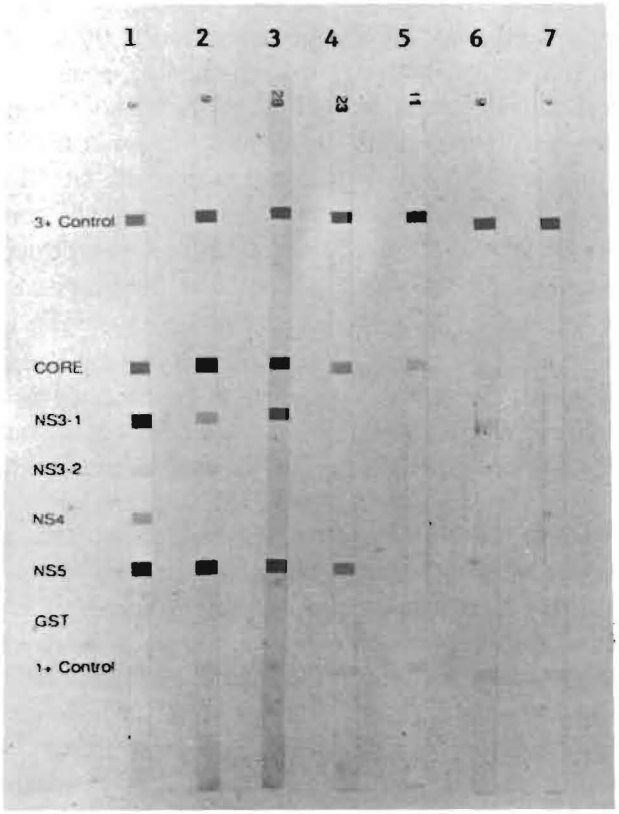

Fig. 2. Examples of the immunoreactivity patterns on immunoblot assay in plasma samples from Thai blood donors; strips 1 to 4 were positive, strips 5 and 6 were indeterminate and strip 7 was negative

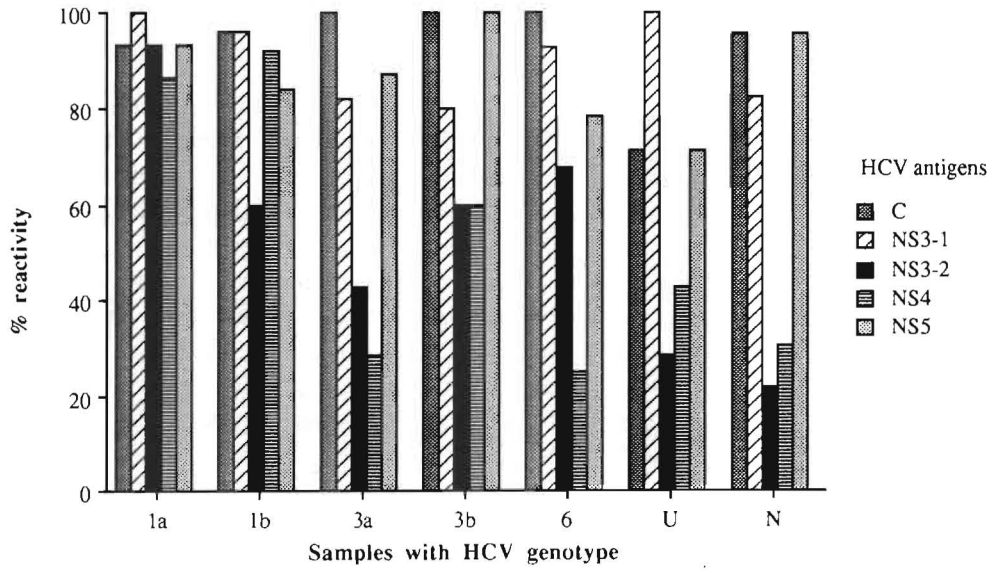

Fig. 3. The immunoreactivity profile of samples containing different HCV genotypes with individual antigens in immunoblot assay;. U, unclassified genotype; N, HCV RNA negative

DISCUSSION

We have studied the profile of immunoblot reactivities of plasma samples from blood donors infected with different HCV genotypes and found that the reactivity on immunoblot assay was influenced by the type of antigen used as well as by the viral genotype. Approximately 81% of plasma samples with repeated HCV EIA positive results were confirmed as positive by immunoblot assay and the result was comparable to the results of the other studies.[12] Notably, all except one of the HCV RNA containing samples also tested positive by immunoblot assay and all of the immunoblot-negative samples were also negative by HCV RNA assay Thus, the immunoblot positivity suggested the presence of a viremic stage. However, about 9% of the immunoblot-indeterminate samples had HCV virions and this suggested that the immunoblot assay alone was not sufficient as the surrogate marker for HCV RNA and HCV RNA assay based on PCR technique should be applied to the samples with either positive or indeterminate immunoblot results to confirm the viremic state. Moreover, long term follow up of EIA-positive blood donors with immunoblot-negative results may be needed to confirm the true status of the non-viremic state.

The HCV antigens used in commercial immunoblot assays such as the HCV BLOT 3.0 assay used in this study are derived from both structural (core) and nonstructural (NS3-1, NS3-2, NS4 and NS5) genes. The incorporation of the core antigen in the EIA and the immunoblot assay significantly increased the sensitivity of the tests because the core gene was highly conserved among different HCV genotypes and the antibodies against the core antigen could be rapidly detected after exposure to HCV.[13,14] In this study, reactivity to core antigen was detected in almost all of the immunoblot-positive samples. The reactivity to core antigen could be detected in all of the samples of genotype 3 and 6 group variants (n=89) and in 95% of genotype 1 samples. There was no different in the reactivity to the core antigen among previously known genotypes ($p > 0.05$). This finding strongly supported the need to incorporate the core antigen into the anti-HCV antibody detection assay due to its high reactivity with samples of any known genotypes. However, only 71% of the samples of the unclassified genotype could react with the core antigen ($p<0.05$ when compared to genotype 1b, 3a and 6 group variants). It may be hypothesized that the lower reactivity to core antigen of the unclassified genotype compared to the other genotypes resulted from differences in the nucleotide sequences in the core gene and the antibodies against the unclassified genotype might not be able to crossreact with the core antigen used in the immunoblot assay. However, the sample size in the unclassified genotype was too small to draw a conclusion. Further study of the nucleotide sequences of this genotype would provide important information for the improvement of the anti-HCV antibody detection assay.

Other nonstructural antigens also contributed to a certain degree to the increase in the sensitivity of the tests. Reactivities with NS3-1 and NS5 antigens were found in 89% and 87% of the immunoblot-positive samples, respectively; and in 90% and 85% of the immunoblot-positive and the HCV RNA positive samples, respectively. The lower rate of immunoreactivities with NS3-1 and NS5 antigens than with the core antigen is due to the lower degree of conservation in the genes encoded the NS3-1 and NS5 antigens. Another possibility is that antibodies against NS3-1 and NS5 antigens may not be well developed in some cases. The immunoreactivities to NS3-1 and NS5 antigens also showed a slight, although not statistically significant, variation among the viral genotypes ($p>0.05$). The immunoreactivities were slightly higher in the samples of genotype 1 than in the samples of non-genotype 1 (Table 2). Interestingly, the samples of genotype 6 group variants reacted well with the NS3-1 (92%) and NS5 (78%) antigens as well as with core antigen (100%), with comparable rates as samples of genotypes 1 and 3. Thus, the detection of antibodies in patients infected with HCV genotype 6 group variants would likely be as efficient as for the other genotypes. The reactivity with NS5 antigen of the samples of the unclassified genotype was, however, rather low compared to genotypes 1, 3 and 6 group variants. The most likely explanation for this low reactivity would be variation in the viral genome.

The reactivities to the NS3-2 and NS4 antigens were significantly affected by HCV genotype as shown in Table 2 ($p \leq 0.001$ and ≤ 0.0001 for NS3-2 and NS4, respectively). Only 52% and 45% of the immunoblot positive samples reacted with NS3-2 and

NS4 antigens, respectively. The reactivity with NS4 antigens was much higher in samples containing HCV genotype 1 (90%) than the non-genotype 1 (32%). This was because the NS4 gene contained regions that were genotype-specific so that samples of the same genotype reacted with this antigen, but the samples of different genotypes could not.[15,16] The NS3-2 antigen could react with 93% of the samples of genotype 1a, but only with 52% of the remaining genotypes. The reactivity with NS3-2 antigen was lowest (28%) in samples of the unclassified genotype.

The reactivities with core, NS3-1 and NS5 were detected in about 90-100%, 94-100% and 57-90%, respectively, of the samples from European patients using RIBA 3.0 (Ortho Diagnostics).[8] This finding was similar to our finding that 97%, 90% and 85% of the Thai samples reacted with the core, NS3-1 and NS5 antigens, respectively. The immunoreactivities to these 3 antigens were also independent on the viral genotype found in Thailand and these antigens were very good as agents for the detection of HCV infection. This study and others also showed that reactivity to NS4 antigen was markedly influenced by the genotype of the virus. As most of the commercial tests including the one used in this study were developed based on the nucleotide sequence of HCV genotype 1, mainly found in North America, samples containing genotype 1 therefore reacted very well with this antigen. However, samples of the other genotypes, such as genotype 3, 6 group variants and the unclassified genotype as shown in this study, did not react well with some of the antigens. Therefore, the incorporation of the NS4 and NS3-2 antigens into the tests may not be beneficial.

The results in this study also showed that the samples of the unclassified genotype reacted rather poorly with most of the antigens except NS3-1 (Table 2). Only 71% of the samples of the unclassified genotype reacted to the core antigen, lower than that of samples of other genotypes (93-100%). This indicates the need to improve the efficiency of the available anti-HCV antibody detection assays, especially in areas where multiple genotypes of HCV are present. Thus, as new or unclassified HCV genotypes are being discovered, the evaluation of the available anti-HCV antibody assays should be performed to ascertain the efficiency of the assays for all HCV genotypes.

In conclusion, this study demonstrated that the assays using the core, NS3-1 and NS5 antigens of HCV could efficiently detect the presence of anti-HCV antibodies in Thai blood donors regardless of the viral genotypes. The samples containing HCV genotype 6 group variants which clustered only in Southeast Asia also reacted well with the core, NS3-1 and NS5 antigens. Therefore, the detection of infection with genotype 6 group variants should not be affected by the current tests. Since the core and NS3-1 and NS5 antigens reacted well to samples from all genotypes found in Thailand, further development of the immunodiagnostic assays should include these antigens. It may not be necessary to incorporate antigens from NS3-2 and NS4 into the diagnostic tests due to their low reactivities with plasma samples from HCV-infected patients in Thailand. The inclusion of NS3-2 and NS4 antigens did not provide any benefits in the detection of anti-HCV antibodies due to variations in the genes among different HCV genotypes. In addition, since the currently available immunoblot assays do not work well on the samples of the unclassified genotypes as shown in this study, further characterization of these genotypes is required for the future improvement of the current anti-HCV antibody assay.

ACKNOWLEDGMENTS

This work was supported by grants from the National Science and Technology Development Agency of Thailand and the Anandhamahidol Foundation. D.K. was supported by Medical Scholar Program of Mahidol University. The authors would like to thank Dr. S. Bejarachandra, Prof. D. Chandanayingyong and Mrs. S. Chinathongthai for providing plasma samples.

REFERENCES

1. Simmonds P, Holmes EC, Cha TA, *et al.* Classification of hepatitis C virus into six major genotypes and a series of subtypes by phylogenetic analysis of the NS-5 region. J Gen Virol 1993; 74: 2391-9.
2. Bukh J, Miller RH, Purcell RH. Genetic heterogeneity of hepatitis C virus: quasispecies and genotypes. Seminars Liver Dis 1995; 15: 41-63.
3. Nakatsuji Y, Matsumoto A, Tanaka E, Ogata H, Kiyosawa K. Detection of chronic hepatitis C virus by four diagnostic systems: first-generation and second-generation enzyme-linked immunosorbent assay, second-generation recombinant immunoblot assay and nested polymerase chain reaction analysis. Hepatology 1992; 16: 300-5.

4. Katayama T, Mazda T, Kikuchi S, *et al.* Improve serodiagnosis of non-A, non-B hepatitis by an assay detecting antibody to hepatitis C virus core antigen. Hepatology 1992; 15: 391-4.

5. McOmish F, Chan S-W, Dow DC, Gillon J, *et al.* Detection of three types of hepatitis C virus in blood donors: investigation of type-specific differences in serologic reactivity and rate of alanine aminotransferase abnormalities. Transfusion 1993; 33: 7-13.

6. Dow BC, Follett EAC, Munro H, *et al.* Failure of 2nd- and 3rd-generation HCV ELISA and RIBA to detect HCV polymerase chain reaction-positive donations. Vox Sang 1994; 67: 236-7.

7. Saldanha J, Mior P. Incidence of hepatitis C virus RNA in anti-HCV-negative plasma pools and blood products. Vox Sang 1996; 70: 232-4.

8. Pawlotsky J-M, Roudot-Thoraval F, Pellet C, *et al.* Influence of hepatitis C virus (HCV) genotypes on HCV recombinant immunoblot assay patterns. J Clin Microbiol 1995; 33: 1357-9.

9. Maggi F, Vatteroni ML, Pistello M, Avio CM, Cecconi N, Panicucci F, Bendinelli M. Serological reactivity and viral genotypes in hepatitis C virus infection. J Clin Microbiol 1995; 33: 209-11.

10. Kanistanon D, Neelamek M, Dharakul T, Songsivilai S. Genotypic distribution of hepatitis C virus in different regions of Thailand. J Clin Microbiol 1997; 35; 1772-6.

11. Songsivilai S, Jinathongthai S, Wongsena W, Tiangpitayakorn C, Dharakul T. High prevalence of hepatitis C infection among blood donors in Northeastern Thailand. Am J Trop Med Hyg 1997; 57; 66-9.

12. Lavanchy D, Mayerat C, Morel B, *et al.* Evaluation of third-generation assays for detection of anti-hepatitis C virus (HCV) antibodies and comparison with presence of HCV RNA in blood donors reactive to c100-3 antigen. J Clin Microbiol 1994; 32: 2272-5.

13. van der Poel CL, Bresters D, Reesink HW, *et al.* Early antihepatitis C virus response with second-generation C200/C22 ELISA. Vox Sang 1992; 62: 208-12.

14. Yeh CT, Han CM, Lo SY, *et al.* Early detection of anti-HCc antibody in acute hepatitis C virus (HCV) by western blot (immunoblot) using a recombinant HCV core protein fragment. J Clin Microbiol 1994; 32: 2235-41.

15. Simmonds P, Rose KA, Graham S, *et al.* Mapping of serotype-specific, immunodominant epitopes in the NS-4 region of hepatitis C virus (HCV): use of type-specific peptides to serologically differentiate infections with HCV types 1, 2 and 3. J Clin Microbiol 1993; 31: 1493-503.

16. Bhattacherjee V., Prescott LE, Pike I, *et al.* Use of NS-4 peptides to identified type-specific antibody to hepatitis C virus genotypes 1, 2, 3, 4, 5 and 6. J Gen Virol 1995; 76: 1737-48.