J Microbiol Immunol Infect
2000;33:149-153

EJ072200000149

Genotypic analysis of HCV infection in Kinmen

Yi-Wen Liou[1], Pesus Chou[1,2], Yi-Ming A Chen[1,3]

[1]Division of Preventive Medicine, Institute of Public Health; [2]Community Medicine Research Center; and [3]AIDS Prevention and Research Center, National Yang-Ming University, Taipei, Taiwan, ROC

Received: December 20, 1999 Revised: February 16, 2000 Accepted: March 24, 2000

In this report, stratified random sampling of an epidemiological study population from the town of Kin-Hu in the Kinmen Islands was used to create a subpopulation of 832 individuals. Two enzyme immunoassays (EIA) were used for antibody testing including Abbott's hepatitis C virus (HCV) EIA 2nd Generation and a synthetic peptide-EIA, NANBASE C-96-EIA, based on the locally predominant strain of HCV. In addition to RIBA and DBL immunoblot assays, reverse transcription-polymerase chain reaction (RT-PCR) was employed to confirm HCV infection. Results showed that 20 of 832 (2.4%) adults in Kinmen had HCV infection. In terms of genotype distribution, 31.3% (5/16) were infected with both 1a and 1b genotypes, 25.0% (4/16) only with the 1b genotype, and 43.8% (7/16) with the 2a genotype. Through comparative analysis of RT-PCR, RIBA, and DBL results, we found that the sensitivities of RIBA and DBL could safely be increased by modifying the definition of a positive case. If the presence of a reactive band with ≥ 2+ antibody reactivity to core protein is accepted as positive for overall RIBA or DBL testing, sensitivity is increased without adversely effecting specificity.

Key words: Genotype, hepatitis C virus, immunoblot assay, Kinmen, reverse transcription-polymerase chain reaction (RT-PCR)

Hepatitis C virus (HCV) infection is the major cause of non-A, non-B hepatitis in the world [1]. Among people infected with HCV, approximately 80% become chronic and more than 20% develop cirrhosis [2,3]. Liver failure or hepatocellular carcinoma is the major cause of death among patients with HCV infection [4,5]. The diagnosis of HCV infection is based on the detection of anti-HCV antibodies in serum, generally by means of enzyme immunoassays (EIA). Since the first-generation HCV-EIAs lacked sensitivity and specificity, confirmatory assays based on immunoblotting were developed [6, 7]. Since then, EIAs have been improved and the assays available today are both sensitive and specific [8,9]. Nonetheless, immunoblot tests are still used for confirmation in most laboratories [10]. In addition to serologic assays, polymerase chain reaction (PCR)-based assays have been developed and are capable of detecting minute amounts of HCV RNA in patients' serum or plasma [11].

In Taiwan, a locally manufactured HCV-EIA, NANBASE C-96 (C-96-EIA), has been employed for screening in blood donation centers since 1992. The implementation of the blood-screening program for HCV infection has dramatically decreased the rates of post-transfusion hepatitis in Taiwan [12]. Since the amino acid sequences of the recombinant proteins and synthetic peptides used in C-96-EIA were deduced from a local HCV clone [13], results may differ somewhat from Abbott's HCV-EIA Second Generation (AB-EIA), an internationally accepted test deduced from a different, internationally more common strain of HCV. The goal of this study is to compare the sensitivity and specificity of these two EIAs in a population in this region. Confirmatory tests, including two immunoblot assays (RIBA and DBL) and reverse transcription-polymerase chain reaction (RT-PCR), were used to verify the results from the two EIAs.

Materials and Methods

Study population and sample collection

Data and samples were collected from an adult population in the town of Kin-Hu in the Kinmen Islands by Yang-Ming Crusade, a team of medical student volunteers organized by the Institute of Public Health of National Yang-Ming University, in collaboration with the Kinmen Health Center during 1991 to 1993. The venipuncture rate was 74.42% (3317 of 4475 residents).

In the first stage of the study, 100 people from each

Corresponding author: Professor Yi-Ming Chen, Institute of Public Health, National Yang-Ming University, No. 155, Section 2, Li-Noun Street, Taipei 112, Taiwan, ROC.

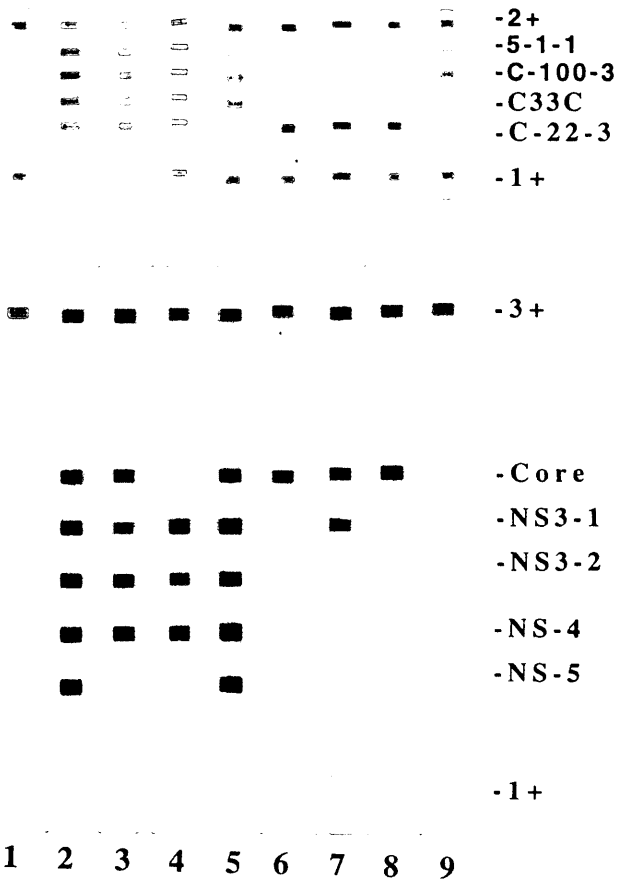

Fig. 1. Immunoblot assay test strip from RIBA (top) and DBL (bottom) tests. The serum samples used in both strips were as follows: lane 1: negative serum control; lane 2: positive serum control; lanes 3-9: actual test results with serum samples from the study population.

age and sex-specific group were randomly selected. All residents more than 80 years old were included due to their limited numbers. In total, 1,227 adults over 30 years old were selected for the study. Among them, serum samples from 832 persons were available for analysis. In the second stage of the study, all individuals who had been tested positive by HCV-EIAs were informed and asked to participate in the follow-up study. Serum samples were collected for confirmatory tests and RT-PCR in 1994.

EIAs

Two EIAs were used for anti-HCV antibody testing, NANBASE C-96 (C-96r, General Biological Co., Taiwan) and Abbott's HCV-EIA 2nd Generation (AB-EIA, Abbott Diagnostics, North Chicago, IL), according to instructions provided by the manufacturers.

Immunoblot assays

Two immunoblot assays were used as HCV confirmatory tests: RIBA 2.0 HCV Strip Immunoblot Assay (Chiron Corporation, Emeryville, CA) and DBL HCV Blot 3.0 Test (Diagnostic Biotechnology Laboratories, Singapore). There are two control bands on the RIBA HCV strip: a level 1 control band on the bottom and a level 2 control band on the top. Between the control bands are four other bands. If two of the four bands (5-1-1, c100-3, c33c, c22-3) are darker than the level 1 control band, then the test is positive (Fig. 1 upper panel). The DBL test strip is very similar, except that there are a total of seven bands (1+, NS-5, NS-4, NS3-2, NS3-2, Core, and 3+, Fig. 1, lower panel).

Reverse transcription-polymerase chain reaction (RT-PCR)

RT-PCR was also used for HCV infection confirmation as described [14]. In the first PCR, positive test results were defined as the presence of a DNA of 272bp. In the second PCR, positive results were defined as the presence of a DNA of 57bp for genotype 1a cases, 144bp for genotype 1b cases, 174bp for genotype 2a cases, and 123bp for all other genotype cases [14,15].

Protocol

First an AB-EIA test was performed once on all samples. Those that scored above the cut-off value were tested again. Only those that tested above the cut-off value twice were considered as positive-unconfirmed cases. This process was then repeated on all samples using the C-96-EIA test. The three confirmatory tests (RIBA and DBL immunoblot assays and RT-PCR) were then used for the samples that tested positively twice in AB-EIA, C-96-EIA, or both.

Statistical analysis

All basic information was stored using Dbase III Plus software. Student's t-test and chi-square test were used for univariate analysis.

Results

As shown in Table 1, among 832 people randomly selected from Kin-Hu, 21 (2.52%) were anti-HCV-antibody positive according to AB-EIA. Prevalence was greatest among persons age 70 or older (5.56%), particularly among males age 70 or older (7.89%). Because of the small number of total positive cases, these results were not statistically significant and no further analysis of age as a determining factor was possible.

AB-EIA is an internationally accepted standard

Table 1. Age and sex-specific positive rates of anti-HCV antibody in Kin-Hu, Kinmen

Age (year)	Prevalence (%)[a]					
	Male		Female		Total	
30-39	3/39	(3.30)	1/103	(0.97)	4/196	(2.04)
40-49	1/79	(1.27)	2/88	(2.27)	3/167	(1.80)
50-59	0/89	(0)	4/72	(5.55)	4/161	(2.48)
60-69	0/66	(0)	1/80	(1.25)	1/146	(0.68)
≥70	6/76	(7.89)	3/86	(3.49)	9/162	(5.56)
Total	10/403	(2.48)	11/429	(2.56)	21/832	(2.52)

[a]Based on data from AB-EIA.

Table 2. A comparison of the sensitivity and specificity of two EIAs for HCV

Categories of EIA	No. of positive cases (N = 832)	Sensitivity[a]	Specificity[b]
AB-EIA	21	75% (15/20)	99.27% (811/817)
C-96-EIA	23	90% (18/20)	99.39% (809/814)
AB-EIA or C-96-EIA	28	100% (20/20)	99.01% (804/812)

Abbreviations: EIA = enzyme immunoassay; HCV = hepatitis C virus

[a]Based on 20 specimens which had been confirmed by immunoblot assays and RT-PCR.

[b]Specimens found negative by test / (total number of specimens minus confirmed specimens).

procedure, while C-96-EIA is based on the locally predominant strain of HCV. As shown in Table 2, 21 serum samples were tested positive by using AB-EIA and 23 by C-96-EIA. Both tests missed some potential cases found by the other. Together, these two tests identified 28 (3.37%) potential HCV infections among 832 adults in the study population.

Subsequently, the 28 positive cases were tested using RIBA and DBL immunoblot assays, and 13 cases were found to be positive according to at least one test (Fig. 1). Furthermore, among 15 patients whose antibody reactivity could not be confirmed by immunoblot assays, seven were found to be positive using RT-PCR. In total, 20 of 832 (2.4%) adults in Kin-Hu had HCV infection. Among them, AB-EIA had detected 15 (75% sensitivity) and C-96-EIA had detected 18 cases (90% sensitivity). The difference between the sensitivity rates was not statistically significant ($p = 0.0934$). In terms of specificity, AB-EIA was 99.27% and C-96-EIA was 99.39% (Table 2).

Sixteen fresh serum samples were available for HCV genotype analysis. The results showed that five (31.3%) were infected with both genotypes 1a and 1b, four (25%) were infected with genotype 1b and seven (43.8%) were infected with genotype 2a.

Both manufacturers indicated that criteria to be used for a positive case using RIBA and DBL tests is the presence of two or more bands in one lane that are darker than the 1+ control band in the same lane. When the patterns of the antibody reactivity to different HCV antigens in the immunoblot assays were examined, there were two cases that were missed by both RIBA and DBL and three cases that were missed by either one or the other. All five of these cases appeared as indeterminate in the immunoblot assays. As shown in Table 3, four of five cases had ≥ 2+ antibody reactivity to HCV core protein in both tests. If the presence of one reactive band with ≥ 2+ antibody reactivity in the immunoblot assay was included as one of the definitions of HCV infection, sensitivity was increased without having a negative effect on specificity.

As shown in Table 4, among 28 EIA-positive cases, 13 cases (46.2%) were confirmed by either RIBA or DBL immunoblot assay. Since there were 20 HCV cases confirmed by immunoblot assays and RT-PCR, the detection rate for immunoblot assays used in this study was 65%. When the new criteria for positive cases using immunoblot assay were adapted, the detection rate for RIBA was increased to 55%, for DBL to 60%, and 85% for RIBA and DBL combined.

Discussion

In this study, C-96-EIA appeared to be more sensitive than AB-EIA. This is probably because C-96-EIA was designed based on isolates of the locally predominant HCV strain. The C-96-EIA kit costs roughly half of that for AB-EIA. Both tests missed some HCV-infected cases that were found by the other and that were later

Table 3. Cases in which modified criteria for the HCV immunoblot test would have changed results from indeterminate to positive

Case no.	Subtype	Applicable protein readings of modified criteria for immunoblots	
		RIBA	DBL
1769	1a & 1b	Positive	In[a] (Core 4+)
1805	1b	In (c22-3 3+)	Positive
2014	2a	In (c22-3 2+)	In (Core 2+)
3679	1a & 1b	In (c100 1+)	In (Core 2+)
1405[b]	2a	Positive	In (Core 1+)

[a]Indeterminate.
[b]This case was still scored as indeterminate according to the new criteria.

Table 4. A comparison of two immunoblot tests (RIBA and DBL) and a RT-PCR assay

Immunoblot assays	Positive rate in HCV-EIA-positive cases	Detection rate based on	
		Old criteria	New criteria[a]
RIBA	32.1% (9/28)	45% (9/20)	55% (11/20)
DBL	32.1% (9/28)	45% (9/20)	60% (12/20)
RIBA & DBL	46.2% (13/28)	65% (13/20)	85% (17/20)

[a]The new criteria were modified from the manufacturer's criteria (old criteria) and regard serum samples with 2+ or above 2+ anti-core antibody reactivity as confirmed cases in the immunoblot assays.

confirmed, hence it would be ideal to use both tests for such screening procedures. Since AB-EIA is an international standard in epidemiological research, its continued use worldwide encourages further comparative analysis.

The third generation (3.0) AB-EIA replaced the second generation (2.0) AB-EIA in 1995. However, when the study was conducted, only the 2.0 AB-EIA was available. According to the manufacturer, the 3.0 AB-EIA has a higher sample/cut-off ratio than the 2.0 AB-EIA. In addition, according to the manufacturer's evaluation report, a known confirmed anti-HCV positive sample from Edinburgh, which was negative in the 2.0 assay, was detected by the 3.0 assay (personal communication, Abbott Laboratory). Further studies are needed to determine whether the 3.0 AB-EIA can detect those samples missed by the 2.0 assay in the present study.

In previous studies in Taiwan, the major genotype of HCV infection among blood donors and patients with hepatocellular carcinoma was genotype 1b, and very few of them were infected with genotype 1a [16,17]. In this study, although more than half of the HCV infections in Kin-Hu were genotype 1b, there were five (31.3%) cases of genotype 1a infection. Further study is needed to elucidate the distribution and risk factors associated with different genotypes in the Kinmen Islands.

As shown in Table 3, all five cases that were missed by RIBA or DBL and confirmed by RT-PCR appeared to be indeterminate in the immunoblot assays. This suggests that a problem might exist in the criteria used in the immunoblot assays for determining positive versus indeterminate results. The criteria of a positive finding in RIBA and DBL tests are the presence of two or more bands that are darker than the 1+ control band in the same strip. If only one band is darker than the 1+ control it is considered indeterminate, and if no bands are darker than the 1+ control it is considered negative. Among the five cases mentioned above, the single darker bands that did appear were for core proteins (C-100-3 or C-22-3 for RIBA, and Core for DBL). This suggests that it might be possible to modify the criteria for both tests such that one darker band with $\geq$ 2+ antibody reactivity for a core protein be considered positive and not indeterminate. The sensitivity of both immunoblot assays would thus be increased without adversely effecting specificity. Further study with a larger sample size is needed to confirm this.

Acknowledgments

We would like to thank members of Yang-Ming Crusade and Director Gin-Son Wu of the Kinmen Health Center for their help in collecting data and serum samples; Cheng-Tau Chen, Chiou-Yu Lin and Swei-Song Chiang, for their technical assistance; and Mr. Christopher Chalfant for editing the manuscript . Dr. Yi-Ming Chen is funded by an award from the Medical Research and Advancement Foundation in memory of Dr. Chi-Shuen Tsou.

References

1. Alter HJ, Purcell RH, Shih JW, Melpolder JC, Houghton M, Choo QL, Kuo G. Detection of antibody to hepatitis C virus in prospectively followed transfusion recipients with acute and chronic non-A non-B hepatitis. N Engl J Med 1989;321:1494-500.
2. Di Bisceglie AM, Goodman ZD, Ishak KG, Hoofnagle JH, Melpolder JJ, Alter HJ. Long-term clinical and histopathological follow-up of chronic post-transfusion hepatitis. Hepatology 1991;14:969-74.
3. Seeff LB. Natural history of viral hepatitis, type C. Semin Gastrointest Dis 1995;6:20-7.
4. Fattovich G, Giustina G, Degos F, Tremolada F, Diodati G, Almasio P, Nevens F, Solinas A, Mura D, Bonetti P, Fuschi P, Brouwer JT, *et al.* Morbidity and mortality in compensated cirrhosis type C: a retrospective follow-up study of 384 patients. Gastroenterology 1997;112:463-72.
5. Poynard T, Bedossa P, Opolon P. Natural history of liver fibrosis progression in patients with chronic hepatitis C. Lancet 1997;349:825-32.
6. Weiner AJ, Truett MA, Rosenblatt J, Han J, Quan S, Polito AJ, Huo G, Choo QL, Houghton M. HCV testing in low-risk population. Lancet 1990;336:695.
7. Lok ASF, Ma OCK, Chan TM, Lai CL, Chung HT, Ng CPL, Lam JSC. Overestimation of the prevalence of antibody to hepatitis C virus in retrospective studies on stored sera. Hepatology 1991;14:756-62.
8. Chaudary RK, McLean C. Detection of antibody to hepatitis C virus by second-generation enzyme immunoassay. Am J Clin Pathol 1993;99:702-4.
9. Farci P, London WT, Wong DC, Dawson GJ, Vallari DS, Engle R, Purcell RH. The natural history of infection with hepatitis C (HCV) in chimpanzee: comparison of serologic responses measured with first and second-generation assays and relationship to HCV viremia. J Infect Dis 1992;165:1006-11.
10. Van der Poel CL, Cuypers HT, Reesink HW, Weiner AJ, Quan S, Di Nello R, Van Boven JJ, Winkel I, Mulder-Folkerts D, Exel-Oehlers PJ, Schaasberg W, Leentvaar-Kuypers A, Polito A, Houghton M, Lelie PN. Confirmation of hepatitis C virus infection by new four-antigen recombinant immunoblot assay. Lancet 1991;337:317-9.
11. Sayers MH, Gretch DR. Recombinant immunoblot and polymerase chain reaction testing in volunteer whole blood donors screened by a multi-antigen assay for hepatitis C virus antibodies. Transfusion 1993;33:809-13.
12. Wang YJ, Lee SD, Hwang SJ, Chan CY, Chow MP, Lai ST, Lo KJ. Incidence of post-transfusion hepatitis before and after screening for hepatitis C virus antibody. Vax Sang 1994;67: 187-90.
13. Chen PJ, Lin MH, Tai KF, Liu PC, Lin CJ, Chen DS. The Taiwanese hepatitis C virus genome: sequence determination and mapping the 5' termini of viral genomic and antigenomic RNA. Virol 1992;188:102-13.
14. Okamoto H, Sugiyama Y, Okada S, Kurai K, Akahane Y, Sugai Y, Tanaka T, Sato K, Tsuda F, Miyakawa Y, Makoto M. Typing hepatitis C virus by polymerase chain reaction with type-specific primers: application to clinical surveys and tracing infectious sources. J Gen Virol 1992;73:673-9.
15. Simmonds P, Alberti A, Alter HJ, Bonino F, Bradley DW, Brechot C, Brouwer JT, Chan SW, Chayama K, Chen DS, Choo QL, Colombo M, *et al.* A proposed system for the nomenclature of hepatitis C viral genotypes. Hepatology 1994;19:1321-4.
16. Wu CH, Lee MF, Kuo HS. Distribution of hepatitis C virus genotypes among blood donors in Taiwan. J Gastroenterol Hepatol 1997;12:625-8.
17. Chen CH, Shen JC, Wang JT, Huang GT, Yang PM, Lee HS, Lee CZ, Chen DS. Genotypes of hepatitis C virus in chronic liver disease in Taiwan. J Med Virol 1994;44: 234-6.