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Hepatitis C Virus Nonstructural 3 Protein: Recombinant NS3 Protein of the Thai Isolates as an Antigen in a Diagnostic Assay

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SUMMARY **Nonstructural 3 (NS3) protein of hepatitis C virus (HCV) is one of the antigens commonly used in diagnostic assays for antibody to hepatitis C virus. However, immune response to the NS3 protein from one genotype may not cross-react with that from other genotypes. In the development of an anti-HCV assay, the NS3 genes from genotypes 1 and 3 commonly found in Thailand were amplified and cloned into a bacterial expression system. These recombinant NS3 proteins were immunogenic and reacted with plasma samples of Thai patients infected with various HCV genotypes. Interestingly, the NS3 proteins from the Thai genotypes could react with 3 plasma samples from HCV infected Thai blood donors, which could not bind to the NS3.1 protein in the commercial HCV immunoblot kit using antigen from HCV genotype 1. This finding supports our prior observation that the appropriate HCV antigens used in a diagnostic assay should be derived from the virus genotypes commonly found in that geographical region.**

Hepatitis C virus (HCV) infection is a major global public health problem. The diseases caused by HCV range from acute hepatitis and chronic hepatitis to cirrhosis and hepatocellular carcinoma. In Thailand, approximately 1.5% of the healthy population is infected with this RNA virus.[1] The majority of HCV-infected patients can not eliminate the virus and gradually develop chronic liver diseases. Currently, there is no vaccine available for HCV. The screening of blood and blood products for antibody to HCV is an effective means for preventing the transmission of HCV.

As a result of its error-prone RNA polymerase activity and the lack of repair mechanisms, great genetic heterogeneity is found distributed throughout the HCV genome.[2] At least 6 major genotypes of HCV were characterized and were unevenly distributed in different regions of the world.[3,4] Genotype 3a is found predominantly in Thailand followed by genotypes 1 and 6.[5] On the contrary, genotype 1 is the major genotype in USA and Japan. This genetic heterogeneity causes problems in the immunodiagnosis of HCV infections due to antigenic variations among different HCV genotypes. Even within the same genotype, genetic variations were found among isolates from different geographical regions.

The 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*. The commercial assays for HCV infection diagnosis were manufactured based on molecular information of HCV genotype 1.[6] This is problematic when applying the test in a region where other genotypes of HCV are dominant. Our group has previously reported that commercial assays could not detect HCV antibodies in some Thai patients who were infected with HCV. For example, the immunoreactivities to the NS3.2 and NS4 antigens in the commercial

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HCV BLOT 3.0 assay (Genelabs Diagnostics, Singapore) were significantly less detected in samples from patients infected with genotypes 1b, 3, 6 or the unclassified HCV genotypes, compared to those of genotype 1a.[7] The immunoreactivities to the core, NS3.1 and NS5 antigens were relatively less dependent on viral genotypes. A study using blood samples from HCV-infected European patients which were tested with the commercial immunoassay RIBA 3.0 (Ortho Diagnostics) also showed similar results.[8]

During the development of an immunodiagnostic assay for HCV infection based on information of HCV serotypes commonly found in Thailand, the core (nucleocapsid) and NS5 antigens of Thai HCV genotypes were expressed and showed strong immunoreactivities to HCV-infected blood samples.[9] In this study, recombinant NS3 antigen, one of the most important proteins for detecting antibodies to HCV, was expressed using genes cloned from HCV genotypes 1 and 3, the most common genotypes found in Thailand. The immunogenicities of these recombinant proteins were evaluated using panels of anti-HCV-positive and anti-HCV-negative plasma samples.

MATERIALS AND METHODS

Forty-six HCV seropositive and 9 seronegative plasma samples were obtained from the Blood Transfusion Center at Siriraj Hospital in Bangkok. Antibodies to HCV were assayed using a third-generation enzyme linked immunosorbent assay (Abbott Diagnostics, USA). The presence of antibodies to individual HCV proteins were determined using a commercial HCV BLOT 3.0 assay (Genelabs Diagnostics). The assays were performed according to the manufacturer's instructions. HCV RNA was detected by reverse transcription-polymerase chain reaction (RT-PCR) amplification using primers in the conserved 5' noncoding and nonstructural 5 regions.[10] The HCV genotype was determined by a reverse hybridization assay as previously described.[5] The HCV serotype was determined using the Murex HCV serotyping 1-6 assay kit (Murex, United Kingdom).

Amplification of HCV NS3 genes

HCV RNA was extracted from plasma samples using QIAamp Viral RNA Mini Kit (Qiagen, USA), according to the manufacturer's instruction. The reverse transcription process was carried out at 42°C for 30 minutes. Nested PCR amplification was performed with a temperature profile of 35 cycles, each consisting of 95°C for 1 minute, 50°C for 1 minute and 72°C for 1 minute, followed by a final extension step at 72°C for 10 minutes. The sequences and positions of the oligonucleotide primers for amplification of the NS3 gene of HCV genotypes 3 and 1 are described in Table 1. Restriction enzyme recognition sites were appended at both ends of inner oligonucleotide primers to facilitate the directional cloning procedure. The PCR product was detected using agarose gel electrophoresis and ethidium bromide staining.

Cloning of HCV NS3 gene into pPROEX-1

A prokaryotic protein expression system based on the 6-his-

Table 1 Sequences of oligonucleotide primers for amplification of the HCV NS3 gene

Primers	Nucleotide sequences (5' to 3')	Position	Polarity
Outer			
NS3-1ab-1L	GCY GTR GGC ATM TTC MGG GC	3526-3545[a]	+
NS3-1ab-2R	TRG GRT CMA RGC TGA ART CG	4386-4406[a]	-
NS3-3a-5L	AYG YBG YVG GSA TCT TYM GG	3542-3561[b]	+
NS3-3a-6R	GGG TCH ARR CTR AAG TCR AC	4402-4421[b]	-
Inner			
NS3-1ab-3L	GGA ATT CAG GCG GTG GAC TTY ATM CCY G	3572-3592[a]	+
NS3-1ab-4R	GCC AAG CTT ACA YGT RTT RCA GTC KAT C	4353-4373[a]	-
NS3-3a-7L	GGA ATT CGY ACC AGR GGT GTV GCR AAR KCC	3572-3594[b]	+
NS3-3a-8R	CTG AAG CTT AGC BAC GTT GCA RTC KAT SAC	4369-4389[b]	-

Note: Y = C or T, R = A or G, M = A or C, B = C, G or T, V = A, G or C, S = G or C, H = A, C or T, K = G or T.
Underlines indicate restriction enzyme recognition sites.
[a]Position according to HCV-1 prototype, [b]Position according to NZL-1 prototype

tidine tag was chosen in order to facilitate purification of the expressed recombinant protein. The NS3 PCR product was purified using the QIAquick PCR purification kit (Qiagen, USA). The purified PCR product and the pPROEX-1 vector (Life technologies, Gibco BRL, USA) were digested with the restriction enzymes *Eco* RI and *Hind* III. The PCR product and vector were ligated at 16°C overnight using T4 DNA ligase (New England Biolabs, USA). *E. coli* strain DH5α frozen competent host cells were then transformed with the ligation reaction and plated onto ampicillin containing agar.

Expression of HCV NS3 proteins

The recombinant clones were screened using either plasmid minipreparation/restriction enzyme digestion or colony-based PCR amplification procedures. Bacterial clones with a DNA insert of approximately 800 bp were subjected to a preliminary assay for the induction of protein expression. In brief, the overnight cultures of recombinant plasmid clones were diluted (1:100) in ampicillin-containing broth and grown at 37°C. Induction of recombinant protein expression was achieved by adding isopropyl-β-D-thiogalactopyranoside (IPTG) into log-phase bacterial cultures to the final concentration of 0.6 mM. The culture fluid sample (1 ml) was taken at 1 hour interval for a period of 6 hours after induction. The bacterial cells were pelleted and resuspended in 1x phosphate buffered saline (PBS) and 2x loading buffer and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The NS3 protein was detected by Coomassie blue dye staining and an immunoblot assay with plasma samples from HCV-infected blood donors. The expected size of the recombinant fusion NS3 protein was about 30 kDa.

Immunoreactivity of NS3 proteins

The immunoreactivity of the recombinantly expressed NS3 proteins was demonstrated by western blot analysis using anti-HCV-positive plasma samples. Briefly, the recombinant protein was electrophoretically blotted onto a nitrocellulose membrane and the membrane was blocked in 0.05% PBS-Tween containing 5% fetal calf serum (FCS). The membrane was then washed, and incubated with either anti-HCV-positive or anti-HCV-negative plasma samples (1:100 dilution). The bound antibodies were detected using alkaline phosphatase-labeled anti-human IgGAM antibody (at 1:2,000 dilution). The color was developed with 5-bromo-4-chloro-3-indolyl phosphate-nitro blue tetrazolium (BCIP/NBT) as enzyme substrate.

Nucleotide sequence determination

Nucleotide sequence determination was performed by direct cycle sequencing from the PCR product and from the recombinant clones. The PCR product was electrophoresed in 1.5% low melting point agarose gel and the DNA band was excised and purified using the QIAquick gel extraction kit. Plasmid DNA was extracted using a commercial Qiagen column. The sequencing reaction was carried out using the BigDye terminator ready reaction mix (Applied Biosystems Inc, USA), according to the manufacturer's instruction. Fluorescent sequencing signals were detected by electrophoresis in the ABI310 automated DNA sequencer (Applied Biosystems). The nucleotide and deduced amino acid sequences were then compared to those of HCV isolates with known genotypes.

RESULTS

Amplification of NS3 genes

The NS3 genes from plasma samples of a Thai patient infected with HCV genotypes 1 and 3 were successfully amplified using oligonucleotide primers of the respective genotypes as in Table 1. DNA fragments of about 800 bp were gel-purified and cloned into

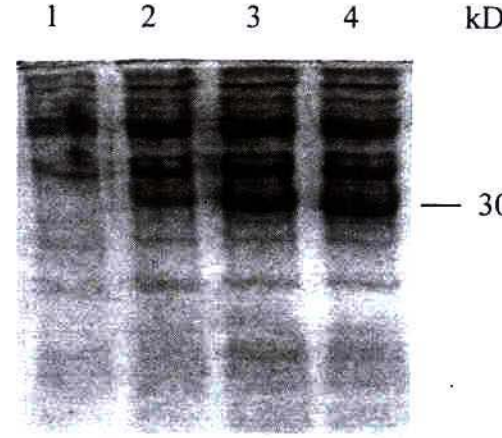

Fig. 1 SDS-PAGE analysis of a recombinant bacterial clone expressing HCV NS3 at different times after induction. Lanes 1-4: bacterial lysate at 0, 1, 2 and 3 hours after induction

pPROEX-1 expression vector and transformed into *E. coli* strain DH5α.

Expression of recombinant NS3 proteins of genotypes 1 and 3

Two screening procedures were used to identify the recombinant HCV NS3 genes. SDS-PAGE analysis and immunoblot assay detected recombinant clones expressing major proteins of approximately 30 kDa, as expected. Induction analysis showed that the intensity of the band increased with time after induction (Fig. 1). One recombinant plasmid was selected and subcloned. The recombinant protein from this clone reacted strongly with plasma samples from HCV-infected Thai blood donors. A similar result was obtained with the cloning and expression of the genotype 3 NS3. The colony-based PCR amplification approach was applied for screening of recombinant *E. coli* containing the NS3 gene, and the recombinant colonies were subjected to further protein expression and immunoblot studies. Several clones expressed immunoreactive NS3 proteins, one of which was chosen for sub-cloning. The recombinant protein from this clone also reacted strongly with plasma samples from HCV-infected Thai patients (Fig. 2).

Immunoreactivity of NS3 proteins from different HCV genotypes

In order to identify whether recombinant HCV proteins from the Thai isolates of HCV genotypes 1 and 3 were immunogenic, immunoblot analysis was carried out using a panel of 43 seropositive samples with known HCV genotypes, and 3 seropositive samples with an unclassified HCV genotype. This panel represented the HCV genotypes 1, 3, 6 and the unclassified genotypes found in Thailand. Genotypes were identified by reverse hybridization technique. In addition, 9 anti-HCV-negative samples were tested in parallel. The re-

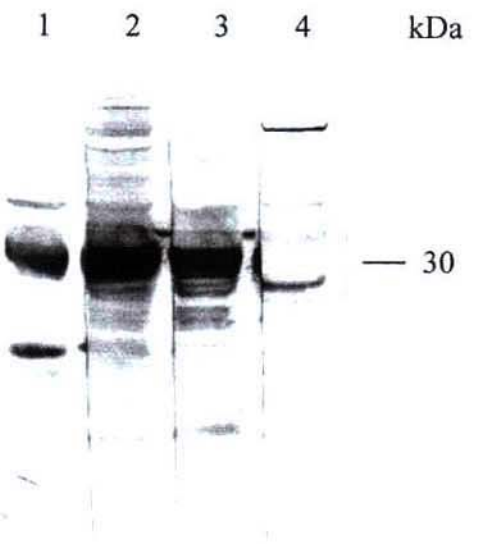

Fig. 2 Immunoblot analysis of recombinant HCV NS3 protein of genotype 3 reacted with blood samples from Thai patients infected with different genotypes of HCV. Lanes 1-3: sample from Thai patients infected with HCV genotypes 1, 3 and 6, respectively. Lane 4: Negative control sample

Table 2 Immunoblot analysis of plasma samples from Thai blood donors infected with various HCV genotypes. The samples were tested with the recombinant NS3 proteins from Thai isolates of genotypes 1 and 3, and with NS3.1 protein in the HCV BLOT 3.0 assay

HCV genotype	No. of reactive samples/total samples tested with		
	NS3 genotype 1	NS3 genotype 3	NS3.1 in HCV BLOT 3.0
1	21/21	18/21	20/21
3	20/20	20/20	18/20
6	2/2	2/2	2/2
Unclassified	2/3	2/3	3/3
Total	45/46	42/46	43/46
Sero-negative	0/9	0/9	0/9

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                                 20                  40                  60
                        *         *         *         *         *         *         *
Thai NS3 type 3     PXEPLSTQTRSPSFSDNSTPPAVPQSYQVGYLHAPTGSGKSTKVPAAYVAQGYNVLVLNPSVAATLGFGS
NZL1 (3a)           .v.t....as............................................................
Thai NS3 type 1                   t...s......cf..ah.................a....k...............a
HCV-1 (1a)          .v.n.e.tms..v.t...s..v....f..ah.................a....k...............a
HCV-H (1a)          .v.n.e.tms..v.t...s.......f..ah.................a.k..k...............a
HCV-J (1b)          .v.sme.tm...v.t...s......tf..ah.................a....k...............a
HCV-T (1b)          .v.sme.tm...v.t...s......af..ah.................a....k...............a
HC-J6 (2a)          .v.s.e.tms..v.t...s.......f..ah.................a....k...............a
HC-J8 (2b)          .v.s.dva..t.....................................as...k...............a

                      80                 100                 120                 140
                       *         *         *         *         *         *         *
Thai NS3 type 3     FMSRAYGIDPNIRTGTRTVTTGAKLTYSTYGKFLADGGCSGGAYDVIICDECHAQDATSILGIGTVLDQA
NZL1 (3a)           ...............n...........................................m..m.......
Thai NS3 type 1     y....h.........v..i...spi.....................i.......st...............
HCV-1 (1a)          y..k.h......s..vs.i...spi.....................mm......st...............
HCV-H (1a)          y..k.h.v....s..vs.i...spi...........a.........mm......st.....s.........
HCV-J (1b)          y..k.h..e...s..vs.i...gpi.....c...............imm.....st.s.t...........
HCV-T (1b)          y..k.h.v....s..vs.i....pi.....................imm.....st.s.t...........
HC-J6 (2a)          y..k.h......s..vs.i...spi.....................mm......st....v..........
HC-J8 (2b)          y..k.h..n...s..v......dsi........i.....aa....i.m.....sv...t...........

                                160                 180                 200
                        *         *         *         *         *         *         *
Thai NS3 type 3     ETAGVRLTVLATATPPGSITVPHSNIEEVALGSEGEIPFYGKAIPIALLKGGRHLIFCHSKKKCDELASK
NZL1 (3a)           .....s.Ss.....................................m.m......s.............m...
Thai NS3 type 1     ....a..v..........v....p.......stt...........leai...................a.
HCV-1 (1a)          ....as.v..........v....p.......stt...........levi...s...............a.
HCV-H (1a)          ....as.v..........v..s.p.......stt...........levi...s...............a.
HCV-J (1b)          ....a..v...............p.......snt............eai...s...............a.
HCV-T (1b)          ....a..v..........v....p....m..snt............eti...s...............a.
HC-J6 (2a)          ....as.v...............a.......stt...........leai...s...............a.
HC-J8 (2b)          .....s.v.........tv.t....m......h............l.fi...s...............aa

                       220                 240
                        *         *         *         *
Thai NS3 type 3     IRGMGLNAVAYYRGLDVSVIPTAGDVVVCATDALMTGFTGDFD
NZL1 (3a)           ls..........s......m..t....................
Thai NS3 type 1     lval.v................s.....v......
HCV-1 (1a)          lval.i................s.....v........y.....
HCV-H(1a)           lval.i................s.....vs
HCV-J (1b)          lt.l...............m..s.....v..............
HCV-T (1b)          lsal.ih............m.as.n...v..............
HC-J6 (2a)          lval.v................s.....v........y.....
HC-J8 (2b)          l...v.......s......m..q.....v........y.....
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Fig. 3 Alignment of deduced amino acid sequences of the recombinant NS3 protein of genotypes 3 and 1, compared to the sequences of other HCV genotypes in the database

sults are shown in Table 2. The recombinant proteins used for analysis were recombinant NS3 proteins from Thai isolates of genotypes 1 and 3, as well as the NS3.1 in a commercial immunoassay for antibodies to HCV. This protein was derived from the Western isolate of genotype 1.

None of the anti-HCV-negative samples reacted with any of the recombinant NS3 proteins of either genotype, showing 100% specificity. Of the 46 anti-HCV positive samples

tested, 45 reacted with genotype 1 NS3 (97.8%), 42 with genotype 3 NS3 (91.3%), and 43 with NS3.1 (93.5%). As with other recombinant HCV antigens used in assays for anti-HCV antibodies, the recombinant NS3 proteins of both genotypes 1 and 3 shall be used in combination. The overall immunoreactivity rate of the Thai NS3 protein was 97.8%, as compared to 93.5% for the Western isolate of genotype 1.

Nucleotide and deduced amino acid sequences of HCV NS3 from genotypes 1 and 3

The nucleotide sequences of HCV NS3 genotypes 1 and 3 were compared and aligned to other known HCV isolates and showed maximal homology to those of isolates HCV-1 (genotype 1a) and NZL1 (genotype 3a), respectively (data not shown). The deduced amino acid sequences, translated from nucleotide sequences with universal codon usage, were aligned to those of other isolates as shown in Fig. 3. Deduced amino acid sequences of the Thai isolates were 92-96% homology to those of isolates of the same genotypes, and about 80-96% homology as compared to those of isolates of different genotypes.

DISCUSSION

The NS3 genes of HCV genotypes 1 and 3 were amplified from HCV-infected Thai patients. Nucleotide and deduced amino acid sequence analysis showed that these isolates were representative of the Thai strains of hepatitis C virus. The level of deduced amino acid sequence similarities was around 90% and 80% when compared to isolates of the same and different genotypes, respectively. Sequence heterogeneity existed even between the Thai genotype 1 and Western genotype 1 (Fig. 3). Therefore, antibodies from patients infected with different HCV genotypes may not cross react with NS3 antigens from other genotypes due to the low homology to heterologous genotypes. This emphasizes the need to utilize antigens from the virus genotypes commonly found in the region for developing an immunoassay, rather than relying on the recombinant protein from other regions.

The NS3 genes of Thai HCV genotypes 1 and 3 were cloned and expressed in a eukaryotic expression vector. Western blot analysis demonstrated that the recombinant NS3 proteins of the Thai isolates could react with the majority of blood samples from HCV-infected Thai blood donors. All but one sample (from a patient infected with HCV of unclassified genotype) reacted with the Thai NS3 protein. This sample was obtained from a healthy blood donor who was tested positive with an anti-HCV ELISA assay but negative by immunoblot. The virus genotype could not be identified by either molecular and serological typing systems. However, this sample had a positive result to NS3 protein in the commercial HCV BLOT 3.0 assay. The reactivity of antibody in this sample to only the NS3.1 antigen may be due to an antigenic variation in the NS3 region, or due to a false-positive result with this commercial test.

Interestingly, the Thai recombinant NS3 proteins could react with 3 samples (1 with genotypes 1 and 2 with genotype 3), that did not react to the NS3.1 protein in the commercial immunoblot assay. This finding supports our prior observations that the appropriate HCV antigens used in the diagnostic assay should derive from the virus genotypes commonly found in the region. In addition, it also confirms the need to incorporate a combination of HCV proteins from various genotypes into the immunodiagnostic assay for anti-HCV antibodies.

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