Applicability of an oligonucleotide probe in radioisotope ^{32}P-based dot blot hybridization for detection of hepatitis C virus in large sample numbers: a preliminary study

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Abstrak

Latar belakang: *Penelitian ini bertujuan untuk merancang dan menganalisis pelacak oligonukleotida apakah dapat diterapkan dalam hibridisasi* dot blot *menggunakan radioisotop ^{32}P untuk mendeteksi virus hepatitis C.*

Metode: *Sampel yang digunakan adalah 46 plasma darah. Plasma diekstraksi untuk mendapatkan RNA genom virus sebagai cetakan reaksi RT-PCR dan amplikon digunakan untuk* nested *PCR. Genom HCV berjumlah 24 diunduh dari* GeneBank *dan penderetan sekuen DNA dilakukan dengan* Software Bio Edit *versi 7.0.9.0. Pelacak oligonuklueotida dirancang berdasarkan daerah lestari genom HCV yang terletak pada sekuen internal di antara 2 primer yang digunakan pada* nested *PCR. Homologi oligonukleotida HCV dianalisis menggunakan teknik* Blast *di* GeneBank. *Radioisotop ^{32}P digunakan untuk melabel oligonukleotida. Oligonukleotida berlabel diaplikasikan untuk produk* nested *PCR menggunakan metode hibridisasi* dot blot. *Konfirmasi hasil amplifikasi dan hibridisasi* dot blot *dilakukan menggunakan metode sekuensing DNA.*

Hasil: *Hasil analisis* Blast *menunjukkan homologi yang tinggi untuk HCV (100%). Hasil* nested *PCR menunjukkan tiga pola fragmen DNA. Tiga pola tersebut masing-masing adalah genotip HCV 1, 2, dan 3. Primer yang digunakan dalam* nested *PCR tidak spesifik dinyatakan dengan adanya tiga fragmen DNA sehingga sulit diinterpretasikan. Hasil hibridisasi* dot blot *menggunakan oligonukleotida yang didesain dalam penelitian ini menunjukkan intensitas dot yang tebal. Semua pola fragmen hasil* nested *PCR menunjukkan hasil positif* dot blot. *Hasil hibridisasi* dot blot *sesuai dengan hasil sekuensing DNA.*

Kesimpulan: *Pelacak oligonukleotida menunjukkan kriteria yang sangat memuaskan secara bioinformatika. Hasil hibridisasi* dot blot *menggunakan ^{32}P menunjukkan intensitas dot yang tebal dan lebih mudah diinterpretasi dibandingkan dengan hasil* nested *PCR.* ***(Med J Indones. 2012;21:71-6)***

Abstract

Background: This study aimed to design and analyze the applicability of an oligonucleotide probe in radioisotope ^{32}P-based dot blot hybridization for detection of hepatitis C virus.

Methods: Forty-six of plasma samples were used. The plasma was extracted to obtain viral RNA genome as template for RT-PCR and the amplicon was used for nested PCR. Twenty-four HCV genomes were retrieved from GeneBank DNA sequence and alignment was performed by Bio Edit Software version 7.0.9.0. An oligonucleotide probe was designed based on a highly conserved region that is located on internal sequence between two primers used for nested PCR. Blast analysis on GeneBank was performed to obtain homology of the oligonucleotide for HCV. The oligonucleotide was then labeled with ^{32}P and dot blot hybridization was applied for nested PCR products. DNA Sequencing was performed to confirm the amplicon and dot blot hybridization results.

Results: Blast analysis showed high homology (100%) for HCV. Nested PCR resulted in three patterns of DNA fragments representing HCV genotypes 1, 2, and 3, respectively. The primers used in nested PCR were not specific and resulted in DNA fragments difficult to be interpreted. Dot blot hybridization using the designed oligonucleotide showed high intensity dots. All nested PCR fragments showed the dot blot positive. The dot blot results were in accordance with DNA sequencing that confirmed three patterns of DNA fragments as different HCV genotypes.

Conclusion: The oligonucleotide showed excellent bioinformatically criteria. ^{32}P-based dot blot hybridization yielded high intensity dots and was easier to be interpreted than nested PCR assay. ***(Med J Indones. 2012;21:71-6)***

Keywords: *Dot blot hybridization, hepatitis C virus, oligonucleotide, radioisotope*

Hepatitis C virus (HCV) infection is an important public health problem. Worldwide, the virus infects 170 million people, and each year, more than 350000 people die from hepatitis C-related diseases primarily chronic hepatitis, cirrhosis, and cellular hepatocarcinoma.[1,2] Hepatitis C virus (HCV) is an envelope-positive sense RNA virus and belongs to the family of Flaviviridae.[3] HCV transmission by inapparent percutaneous exposures caused by cross-contamination from re-used needles and syringes, multiple-use medication vials, and infusion bags has been reported.[4] Moreover, transmission of HCV is less efficient through accidental needlesticks,[4] but the transmission is most efficient through injecting drugs, transfusion or transplantation from infectious donors.[5-7] In developing countries, unsafe medical injections and transfusions are predominant routes of HCV transmissions.[6]

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With the advancement of technologies, diverse methods are available to detect HCV. Serological assays, detecting anti-HCV antibodies and/or HCV antigens, can be performed on any kind of blood samples. In the case of detecting anti-HCV antibodies, the assay can yield false negative results for people with early phase of infection (window period) and immunological dysfunction. Prevention of HCV transmission through blood products will not be achieved if the blood screening only counts on serological assays since the assays can yield false negative results; therefore, complementary testing, such as molecular techniques, is most needed to overcome the limitation of serological assay.

The molecular techniques for HCV detection have been developed including conventional and real time RT-PCR. The conventional RT-PCR is not sensitive.[8] The real time RT-PCR is very sensitive but it is very expensive because of special reagents and thermal cycle machine. In addition, health care services in developing countries including Indonesia rarely have the real time PCR machine. Therefore, in this preliminary study we designed an oligonucleotide probe by a radioisotope ^{32}P-based dot blot hybridization method that can be applied to nested PCR products. The method is highly sensitive,[9] can detect DNA targets of 96 samples in one test and is suitable for screening large sample numbers and for surveillance purposes.

METHODS

The research was carried out at the molecular biology laboratory of Radiation Processing Department, Center for Application of Isotopes and Radiation Technology, National Nuclear Energy Agency, Jakarta.

Clinical specimen and transport

Forty-six plasma samples from suspected patients with HCV infection were obtained from January to December 2009 in the government hospital in Jakarta. To avoid the possibility of RNA or viral destructions caused by transportation, the samples were transported by using a special cool box containing ice packs. After arrival in the laboratory, the samples were immediately stored in -20^{o}C without thawing until used.

Viral RNA extraction

Two hundred microliter of plasma were extracted to obtain the HCV genome RNA by QIAamp Viral RNA Mini Kit (Qiagen) according to manufacturer's instruction, with 50 µL of final elution and immediately used as template for RT-PCR.

RT-PCR and nested PCR

Primers used for RT-PCR and nested PCR from non coding region (NCR) were previously reported by Garson et al.[10] The outer sense and anti-sense primers for RT-PCR were 5' – CCA CCA TAG ATC TCT CCC TGT and 5' – ATA CTC GAG GTG CAC GGT CTA CGA GAC CT , respectively. The RT-PCR reaction mixture was prepared by using one-step RT-PCR kit (Qiagen). The reaction mixture contained 10 µL viral RNA, 1x one-step RT-PCR buffer, 2.5 mM $MgCl_2$, 1x Q solution, 400 µM each of dNTP mix, 0.6 µM each of primers, 2 µL of one step RT-PCR enzyme mix, 10 U RNAse inhibitor and sterile distilled water to a total volume of 50 µL. The RT-PCR was performed in a Master Gradient Thermal Cycler (Eppendorf) with the first step at 50^{o}C for 30 minutes; 35 cycles of 96^{o}C for 30 seconds, 48^{o}C for 45 seconds and 72^{o}C for 1 minute; and extended extension at 72^{o}C for 7 minutes. The nested primers used for the second PCR were 5'- AGA TCT TCA CGC AGA AAG CGT and 5'- CAC TCT CGA GCA CCC TAT CAG GCA GT as inner sense and anti-sense primers, respectively. The reaction mixture of nested PCR consisted of 1x hot star Taq DNA polymerase buffer, 2.5 mM $MgCl_2$, 1x Q solution, 200 µM each of dNTP mix, 0.4 µM each of primer, 1.5 U hot star Taq DNA polymerase (Qiagen), 2 µL of RT-PCR product and sterile distilled water to a total volume of 50 µL. The reactions were run on thermal cycler using the following conditions: 15 minutes at 95^{o}C; 30 cycles of amplification 96^{o}C for 30 seconds, 48^{o}C for 45 seconds, and 72^{o}C for 1 minute; and extended extension at 72^{o}C for 7 minutes. The nested PCR products were analyzed on 1.5% agarose and visualized on ultraviolet transilluminator.

Design of oligonucleotide probe

Twenty-four HCV genomic sequences were retrieved from GeneBank (Figure 1). The sequences were aligned by using Bio Edit version 7.0.9.0.[11] A highly conserved sequence located on internal sequence between two primers used for nested PCR was chosen for oligonucleotide sequence. The reliability and homology (specificity) of the oligonucleotide was analyzed by primer designer-V2.0 serial number: 50053 (scientific & educational software 1991) and GeneBank Blast, respectively.

Oligonucleotide labeling and dot blot hybridization

The oligonucleotide was labeled with radioisotope ^{32}P at its 5' end in 50 µL of reaction mixture with following concentrations: 1x kinase buffer, 1 µM oligonucleotide, 30 µCi of ^{32}P-labeled γ ATP (Hartmann Analytic), 20 U T4 polynucleotide kinase. The mixture was incubated

at 37°C for 30 minutes and terminated by incubating at 72°C for 10 minutes. The dot blot hybridization was performed: 10 µL of nested PCR product was added with 190 µL of the dot buffer (0.4 N NaOH and 25 mM EDTA). The mixture was heated at 100°C and immediately placed on ice, then blotted on Hybond N+ membrane by means of dot blotter (Bio-Rad). The DNA on membrane was fixed by heating at 80°C for 2 hours. The membrane was immersed in hybridization solution (5x SSPE, 5x Denhardt and 0.5% SDS) and shaken at 64°C overnight (16-18 hours). Forty milliliter of the hybridization solution containing ^{32}P-labeled oligonucleotide was reacted with the membrane at 64°C for 1-2 hour. The membrane was washed twice by washing buffer (2x SSPE and 0.1% SDS) at room temperature for 30 minutes, each time followed by a final washing (1x SSPE and 0.1% SDS) at 62°C for 15 minutes. The formed dots on the membrane were detected with autoradiography.

DNA sequencing

To confirm the exactness of specific DNA amplification and dot blot hybridization, three products of nested PCR assays were sequenced. The DNA sequencing results were blasted on GeneBank to obtain the taxonomical reports of the sequences.

RESULTS

Positive nested PCR was defined by a 259 bp DNA band visualized at the right position on 1.5% agarose gel. Of 46 plasma samples, thirty-nine showed 3 different patterns of DNA fragments and 7 showed negative results. Pattern 1 was one specific DNA band (Lane 1, 2, 6, 7, Figure 2), pattern 2 was one DNA band higher in size than the positive control (Lane 8, Figure 2,), and pattern 3 were multi-DNA bands (Lane 3-5, Figure 2). Of 39 samples, thirty-three, five, and 1 showed pattern 3, 1, and 2, respectively. To confirm the amplification products, one of each pattern was sequenced and blasted on GeneBank (Figure 3). The results showed that pattern 1, 2, and 3 were HCV genotypes 2, 1, and 3, respectively. Results as mentioned above indicated that nested PCR primers used in this study are not specific for HCV genotype 1 and 3 because of resulting unspecific DNA band

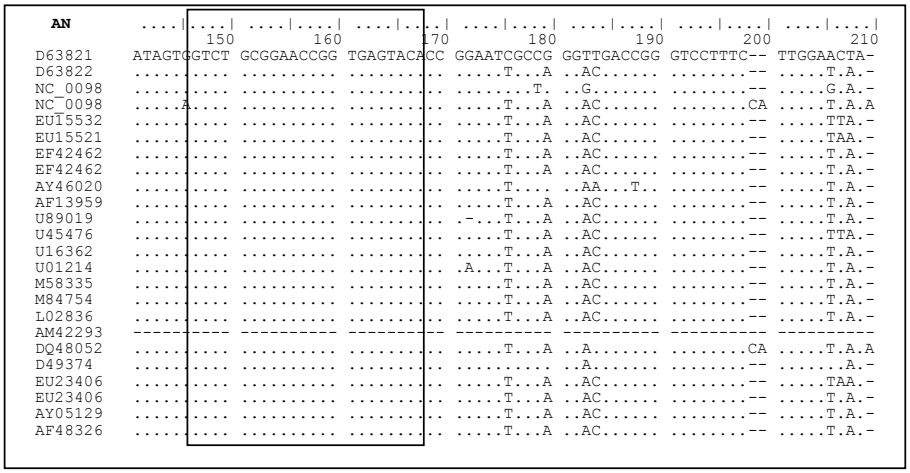

Figure 1. The DNA sequence (GTCTGCGGAACCGGTGAGTACA) from nucleotides 147-168 was used as oligonucleotide probe for dot blot hybridization. The sequence was located between two primers used for nested PCR. AN= accession numbers of HCV genomic sequences that were retrieved from GeneBank

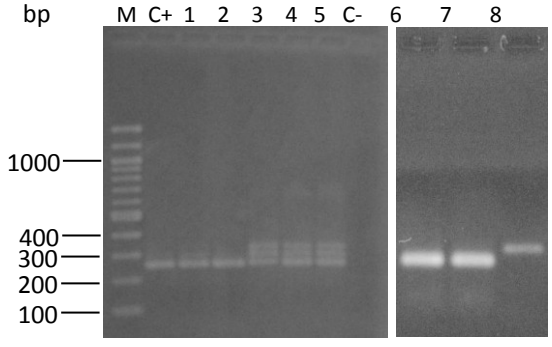

Figure 2. Electrophoresis results of nested PCR assays on 1.5% gel agarose. A specific DNA fragment is defined about 259 bp. M= DNA ladder, C+ and C-= positive and negative controls, respectively, 1-8= eight examples of samples shown, bp= base pairs

A. Taxonomy Report for specific DNA fragment in Lane 7, Figure 2

```
Hepatitis C virus ................................ 100 hits  38 orgs [root]
. Hepatitis C virus genotype 2 ..................  20 hits    4 orgs
. . Hepatitis C virus subtype 2k ................  2 hits     2 orgs
. . . Hepatitis C virus (isolate VAT96) ..........  1 hits     1 orgs
. . Hepatitis C virus subtype 2a ................  11 hits    1 orgs
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B. Taxonomy Report for unspecific DNA fragment in Lane 8, Figure 2

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Hepacivirus .......................   100 hits    4 orgs [root]
. Hepatitis C virus ...............   100 hits    4 orgs
. . Hepatitis C virus genotype 1 ...   93 hits    3 orgs
. . . Hepatitis C virus subtype 1a .   91 hits    1 orgs
. . . Hepatitis C virus subtype 1e .    1 hits    1 orgs
. . . Hepatitis C virus subtype 1b .    1 hits    1 orgs
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C. Taxonomy Report for three multiple DNA fragments in Lane 5, Figure 2

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Hepatitis C virus .....................   100 hits    5 orgs [root]
. Hepatitis C virus genotype 3 ........    17 hits    2 orgs
. . Hepatitis C virus (isolate JK049) .     1 hits    1 orgs
. Hepatitis C virus subtype 4a ........    14 hits    1 orgs
. Hepatitis C virus isolate 06.10.009 .     1 hits    1 orgs
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Figure 3. Blast analysis on GeneBank for three amplification products, specific (A), unspecific (B), and three multiple DNA fragments (C). A, B, and C: samples resulting specific (Lane 7, Figure 2), unspecific (Lane 8, Figure 2), and three multiple DNA fragments (Lane 5, Figure 2), respectively.

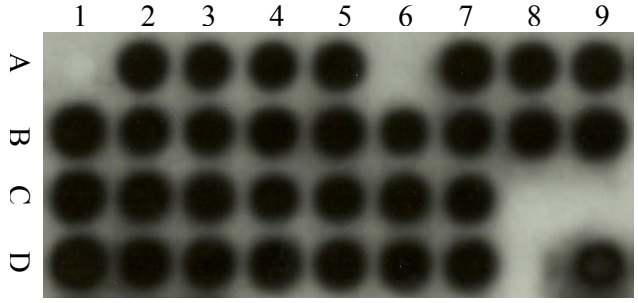

Figure 4. An example of result of dot blot hybridization using ^{32}P-labeled oligonucleotide. Thirty-four tested samples are shown. A1 and A2= negative and positive controls, respectively. Black and clear dots= positive and negative results, respectively.

Table 1. Analysis result of oligonucleotide reliability using primer designer-V2.0 serial number: 50053

Criteria	Criteria Setting	Actual	Meets Criteria
% GC	Min 50, Max 60	59	Yes
Tm (°C)	Min 55, Max 80	77	Yes
No Hairpins	Energy cutoff 0.0 kcal	--	Yes
No 3' Dimers	Reject >= 3 matches at 3' end	2	Yes
No Dimers	Reject >= 7 adj homol bases	6	Yes
No Runs	Reject >= 3 base runs	2	Yes
No 3'GC runs	Reject >= 3 G or C at 3' end	0	Yes

GC= guanine and cytosine, Tm= melting temperature, Hairpins= loop formation on single oligonucleotide

and multi-DNA bands, respectively, leading to the confusing interpretation of nested PCR. Other HCV genotypes 4-6 are not known in this study, they may include one of those patterns or different patterns; therefore, the applicability of nested PCR using primers in this study is questionable to detect all HCV genotypes, specifically.

To overcome the limitation of nested PCR assay, an oligonucleotide probe was designed and evaluated for all nested PCR results. Based on the analysis, the oligonucleotide probe showed excellent criteria (Tabel 1). Technically, hairpins and 3'GC runs on oligonucleotide will influence the specificity, while dimers will influence the sensitivity. The percentage of GC and melting temperature of the oligonucleotide were within normal ranges. Based on Blast analysis on GeneBank, the homology of the oligonucleotide was very high (100%) for HCV (data not shown).

All amplification products of nested PCR assays were probed by radioisotope ^{32}P-labeled oligonucleotide using

the dot blot hybridization method. Of 46 tested samples, thirty-nine samples that showed all three patterns of DNA bands by nested PCR were also dot blot positive. Seven samples with nested PCR negative were also dot blot negative (Figure 4). The results showed that dot-blot hybridization using the oligonucleotide designed in this study could be used for detection of HCV genotypes 1, 2, and 3 specifically, compared with nested PCR showing multi-DNA fragments difficult to be interpreted.

DISCUSSION

RT-PCR followed by nested PCR for detection of HCV was applied for 46 plasma samples. Of these samples, thirty-nine showed the three patterns of DNA bands, namely one specific and one unspecific DNA band, and three multi-DNA bands (Figure 2). Such three patterns of DNA bands were confirmed by DNA sequencing and showed HCV with different genotype (Figure 3). Accordingly, the nested PCR used in this study resulted in unspecific or multi-DNA fragments confusedly to be interpreted. This indicates that primers used in nested PCR are not specific for HCV genotypes 1 and 3. The problem may be caused by different DNA sequences among HCV genotypes/subtypes or HCV quasispecies in one infected patient. HCV isolates can be highly divergent and have been classified into 6 major genotypes and more than 30 subtypes.[11] Like other RNA viruses, HCV circulates *in vivo* as a highly polymorphic population of genetically closely related variants.[12] In consequence, it is difficult to detect or identify all HCV genotypes in a single detection system like using a primer pair of PCR.

As known, PCR relies on the efficient attachment of primers to the DNA target. Mismatches between the primer and the DNA target can influence duplex stability and limit the assay to amplify target sequences. Several factors causing the mismatches are primer length, nature and position of mismatches, hybridization temperature, presence of co-solvents (such as DMSO), and concentrations of primers and monovalent and divalent cations.[12-14] For different HCV genotypes with different genomic RNA sequences, the partial mismatches between primers and DNA target and the presence of insertion/deletion mutation or recombination can also result in unspecific DNA fragments.

To overcome the limitation of nested PCR, an oligonucleotide of 22 nucleotides in length was designed and its applicability was evaluated. Hybridization probes can be applied with lengths from around 20 up to several hundreds of nucleotides. However, longer oligonucleotides lead to more mismatches. To minimize the mismatches, the length of our oligonucleotide was determined in a conserved region (Figure 1). The homology of oligonucleotide by Blast analysis on GeneBank showed 100% homology for HCV (data not shown) indicating technically high specificity of the oligonucleotide for HCV. The oligonucleotide should be unique for the target sequence and fulfill certain criteria such as the length, GC%, annealing and melting temperature (T_m), 5' end stability, 3' end specificity.[14] Annealing the 3' end and/or the 5' end among oligonucleotide segments will interfere the optimal attachment of oligonucleotide to DNA target. An important factor to consider in designing an oligonucleotide probe is the presence of hairpin loops, which reduces the hybridization efficiency by limiting the ability to bind to the DNA target.[15] The oligonucleotide that was designed in this study showed all criteria and it could be applied bioinformatically (Tabel 1).

The oligonucleotide was labeled by ^{32}P isotope and tested for all nested PCR products by dot blot hybridization method. Of 46 samples, thirty-nine were dot blot positive (Figure 4). All nested PCR fragments (specific, unspecific, multi-DNA fragments) were dot blot positive. The results are in accordance with DNA sequencing in that all three patterns of DNA fragments were different HCV genotypes (Figure 3). This indicates that the oligonucleotide designed in this study can detect all nested PCR fragments of HCV genotype 1, 2, and 3. The important differences between nested PCR and dot blot hybridization are that the nested PCR resulted in multi-DNA fragments that are difficult to be interpreted, while the dot blot hybridization showed high intensity dots that are easy to be interpreted. The high intensity results from an autoradiography signal of the radiation emitted by the radioisotope. The ^{32}P has been used widely in dot blot hybridization, Southern blot hybridization, colony and plaque hybridization because it emits high energy β-particles which afford a high degree of sensitivity of detection.[16] For these reasons, numerous researchers have used this approach to detect and identify pathogenic microorganisms.[17-19] Therefore, the dot blot hybridization using oligonucleotide designed in this study is suitable for detection of HCV, particularly for screening of large sample numbers and for surveillance purposes. Other researchers have used the method for large sample numbers for detection and identification of *Dendrobium* species,[20] multi-drug resistant *Mycobacterium tuberculosis*,[9] and Echoviruses.[21] However, the oligonucleotide designed in this study should be evaluated in the future for larger sample numbers to obtain the validity of the specificity and sensitivity.

The dot blot hybridization method that will be developed in the future (application of the method

for RT-PCR instead of nested PCR products) is cost effective compared with nested PCR and real time PCR, giving significant health care-associated cost savings. The conventional PCR is much cheaper than dot blot hybridization, nested PCR and real time PCR; however, it is less sensitive. A limitation of using radioisotope is that a special radioisotope laboratory is needed and the laboratory technicians must have additional knowledge and skills to handle the radioisotope.

In conclusion, an oligonucleotide probe specific for HCV was successfully designed and evaluated by using a ^{32}P radioisotope-based dot blot hybridization method. This method is suitable for detection of HCV for large sample numbers and surveillance purposes. The oligonucleotide showed excellent criteria bioinformatically to be applied for probing HCV from plasma samples. The method yielded high intensity dots that are easier to be interpreted than nested PCR assay with multi-DNA fragments. In future research, the dot blot hybridization will be optimized for RT-PCR instead of nested PCR products and evaluated for large sample numbers.

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