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Comparison of RT-PCR-Dot Blot Hybridization Based on Radioisotope ^{32}P with Conventional RT-PCR and Commericial ELISA Assays for Blood Screening of HIV-1

Perbandingan Uji RT-PCR-Hibridisasi Dot Blot Bertanda Radioisotop ^{32}P, RT-PCR Konvensional dan ELISA Komersial untuk Skrining Darah Terhadap HIV-1

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ABSTRACT

Comparison of RT-PCR-Dot Blot Hybridization Based on Radioisotope ^{32}P with Conventional RT-PCR and Commericial ELISA Assays for Blood Screening of HIV-1. There are many commercial ELISA and rapid test kits that have been used for blood screening; however, the kits can give false positive and negative results. Therefore, RT-PCR (Reverse Transcription Polymerase Chain Reaction) - Dot Blot Hybridization based on radioisotope ^{32}P (RDBR) method was developed in this research, to compare the method with the conventional RT-PCR and commercial ELISA Enzyme-Linked Immunosorbent Assay) kit. This method is efficient for screening of large blood specimens and surveillance study. Eighty seven samples were used and serum of the samples were tested by ELISA to detect HIV-1. The HIV-1 RNA genome was extracted from plasma samples and tested using the RT-PCR and RDBR methods. Of 87 samples that were tested, the rates of positive testing of the RT-PCR, the RDBR, and the ELISA were 71.26%, 74.71%, and 80.46%, respectively. The RDBR (a combination of RT-PCR and dot blot hybridization) was more sensitive than conventional RT-PCR by showing 3.45% in increase number of positive specimens. The results showed that of 9 samples (10.34%) were negative RDBR and positive ELISA, while 4 samples (4.60%) were negative ELISA and positive RDBR. The two methods showed slightly difference in the results but further validation is still needed. However, RDBR has high potential as an alternative method for screening of blood in large quantities when compared to method of conventional RT-PCR and ELISA.

Key words : HIV-1, radioisotope, dot blot hybridization, RT-PCR, ELISA

ABSTRAK

Perbandingan Uji RT-PCR-Hibridisasi Dot Blot Bertanda Radioisotop ^{32}P, RT-PCR Konvensional dan ELISA Komersial untuk Skrining Darah Terhadap HIV-1. Banyak uji berupa kit komersial seperti ELISA dan uji cepat yang digunakan untuk skrining darah ; namun penggunaan kit tersebut dapat memberikan hasil yang salah (*false positive* atau *false negative*). Oleh karenanya, dalam penelitian ini dikembangkan metode RT-PCR – Hibridisasi Dot blot bertanda radioisotop ^{32}P (RDBR) dan kemudian dibandingkan dengan metode RT-PCR konvensional dan kit ELISA komersial. Metode ini efisien untuk skrining sampel darah dalam jumlah banyak dan untuk salah satu penelitian survei. Dalam penelitian ini digunakan 87 sampel darah, dan serum dari sampel diuji untuk mendeteksi HIV-1 dengan ELISA. Metode RT-PCR dan RDBR digunakan untuk mendeteksi HIV-1 dari genom RNA HIV-1 yang diektraksi dari plasma darah. Dari 87 sampel yang diuji dengan metode RT-PCR,

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RDBR dan ELISA menunjukkan persentase hasil positif HIV-1 masing-masing adalah 71,26%; 74,71%, dan 80,46%. Metode RDBR lebih sensitif dari pada RT-PCR konvensional hal ini terlihat dengan bertambahnya jumlah hasil positif sebanyak 3,45%. Data percobaan menunjukkan bahwa 9 sampel (10,34%) adalah hasil negatif RDBR dan positif ELISA, sedangkan 4 (4,60%) sampel negative ELISA dan positif RDBR. Ke dua metode tersebut menunjukkan sedikit perbedaan hasil, namun demikian hasil ini masih memerlukan validasi. Metode RDBR memiliki potensi tinggi sebagai suatu metode alternatif untuk skrining darah dalam jumlah banyak jika dibandingkan dengan metode RT-PCR konvensional dan ELISA.

Kata kunci : HIV-1, radioisotop, hibridisasi dot blot, RT-PCR, ELISA

INTRODUCTION

Blood transfusion is an important part in health care for patients that need blood components because of deficiency in particular blood components or because of too much bleeding. According to WHO, HIV is one of blood burden diseases that has to be screened [1]. However, HIV-1 infections because of HIV contaminated blood transfusions still occur for about 5 – 10% worldwide [2]. The infections can be caused by many factors, such as weak health, rural settings, potentially risky donors, transfusion, and insufficient HIV-1 screening [1, 3]. At emergency situations, blood transfusion without screening is performed because of limitations in test kit and reagent supplies [3].

Enzyme Immunoassay (EIA) and rapid tests are recommended to screen HIV-1 in sera of blood samples for surveillance and diagnostic purpose, due to the fact that both tests are the most accurate and cost-effective [4]. The EIA and rapid tests contain antigens of HIV-1 and they can detect antibodies (Abs) to HIV -1. However, screening by the tests is not possible if the HIV-1 infected persons do not produce HIV-1 specific Abs, or have an immunological dysfunction caused by the course of aggressive diseases, or at the early phase of infection when the HIV-1 specific Abs has not been produced yet [5, 6]. In such conditions, serological diagnosis of HIV-1 infection can yield false negative tests so that the HIV-1 transmission to naïve individuals could occur.

The molecular techniques for HIV-1 detection have been developed including conventional and real time RT-PCR. Both assays are often used as routine tests for variety number of samples. The conventional RT-PCR is not sensitive, while the real time RT-PCR is very expensive because it needs, special thermal cycle machine, and almost all health care services in developing countries including Indonesia such machine is not available yet. The real time RT-PCR reagents are also very expensive especially for large samples. For these reasons, the assessment by using RDBR method and comparison with the conventional RT-PCR and commercial ELISA kit has tobe carried out. The RDBR is not only a highly sensitive method [7] but can also probe the DNA targets for 96 samples in one test; thus, the RDBR is a suitable method for screening of larger blood samples and for surveillance purpose.

MATERIALS AND METHODS

Clinical Specimen and ELISA Test. Eighty-seven of plasma samples were used in this study. All samples were tested by Vironostika HIV Uniform II Ag/Ab (biomerieux). The kit can detect both HIV-1 antigen and anti HIV-1 Ab simultaneously.

Viral RNA Extraction. The HIV-1 RNA were extracted and purified by QIAamp Viral RNA Mini Kit (Qiagen) with 50 µL of final elution. The eluate containing viral RNA was directly used for RT-PCR.

RT-PCR. In RT-PCR reaction, forward [5′-CAG CAT TAT CAG AAG GAG CCA C-3′] and reverse [5′-TCT GCA GCT TCC TCA TTG ATG G-3′] primers were used (8). The

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RT-PCR was performed in 50 µl of reaction mixture with the following compositions: 1x OneStep RT- PCR buffer, 2.5 mM $MgCl_2$, 1x Q solution, 400 µM dNTP mix (dGTP, dCTP, dATP, and dTTP), 0.6 µM of each primer, 10 U RNAase inhibitor, 2.0 µl one step RT-PCR enzyme mix polymerase (Qiagen), and 15 µl extracted viral RNA. The RT-PCR was performed with the following conditions: 37ºC for 30 min; 95ºC for 15 min; 40 cycles of 94ºC for 30 sec, 56ºC for 30 sec, and 72ºC for 45 sec; 72^0C for 7 min. The PCR products were analyzed on 1.5% agarose gel and visualized in ultraviolet light.

Oligonucleotide and the Radioisotope ^{32}P Labeling. An oligonucleotide (TDG GRG GAC AYC ARG SAG CHA TRC A) specific for envelope gene of HIV-1 was used in this study. The oligonucleotide was labeled with radioisotope ^{32}P at its 5′ end in 50 µl of reaction mixture with the following concentrations: 1x kinase buffer, 2 µM oligonucleotide probe, 30 µCi of ^{32}P-labeled gamma ATP (Perkin-Elmer), 20 U T4 polynucleotide kinase. The solution then was incubated at 37ºC for 30 min and and finally at 72ºC for 10 min.

The Radioisotope ^{32}P-Based Dot Blot Hybridization. The RDBR was performed by the following procedure: 20 µl of RT-PCR product that was added with 180 µl of the dot buffer (0.4 N NaOH and 25 mM EDTA). The solution was heated at 100^0C and immediately placed on ice. The DNA was blotted on membrane by the dot blotter (Bio-Rad). The blotted membrane was heated at 80ºC for 2 h for the fixation of the DNA. The membrane was soaked in hybridization solution (5x SSPE, 5x Denhardt and 0.5 % SDS) at 50ºC for overnight (16-18 h). Forty milli liter of the hybridization solution containing 2 µM of the ^{32}P-labeled oligonucleotide probe was reacted with membrane at 50ºC for 1-2 h. The membrane was washed by the washing buffer (2x SSPE and 0.1 % SDS) at room temperature twice for 30 min each, followed by final washing (1x SSPE and 0.1% SDS) at 50ºC for 15 min. The dots that were formed on the membrane were detected by autoradiography.

RESULTS

In this study, conventional RT-PCR positive was defined as a 115-bp DNA fragment that is visualized at the right position on 1.5% agarose gel (Figure 1). The RT-PCR showed specific amplification of

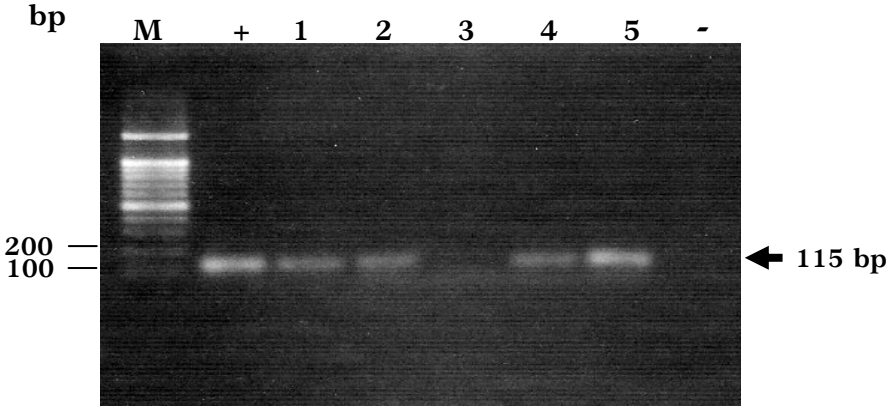

Figure 1. Analysis of RT-PCR products on 1.5% agarose gel. 1-5: the five examples for the RT-PCR positive results showing a 115 bp DNA bands. M: DNA ladder. -: negative control. +: positive control. bp: base pair.

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one expected band (Figure 1). The applicability of the RDBR assay to clinical specimens was evaluated against 87 plasma samples (Figure 2). Of 87 plasma samples, the RT-PCR, the RDBR, and the ELISA showed that the rates of positive testing for HIV-1 are about 71.26%, 74.71%, and 80.46%, respectively. Three RT-PCR negative samples were the RDBR positive; thus, the RDBR represents an increase of 3.45% in number of positive specimens (Table 1). None of the RDBR negative samples showed RT-PCR positive. Contrast results were found while comparing the RDBR to the ELISA (Table 1). In this case, 9 RDBR negative samples were ELISA positive, representing an increase of 10.34% in number of positive samples by the ELISA, and 4 ELISA negative samples were the RDBR positive, representing an increase of 4.60% in number of positive samples by the RDBR assay.

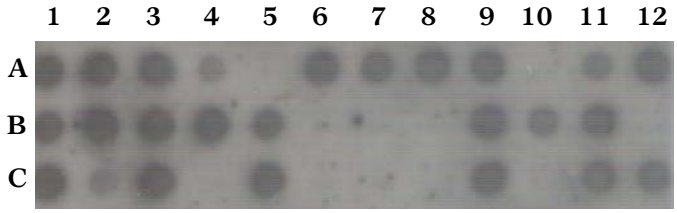

Figure 2. Results of the RDBR method. Positive tests showed the black dots. A2 : positive control, C10 : negative control.

DISCUSSIONS

In any laboratory, a method to prevent the HIV-1 transmission is a crucial need. Nowadays, ELISA and rapid tests are the first and/or screening assays for diagnosing HIV-1. Indeterminate diagnosis test has to be confirmed by Western blot. The rapid test and ELISA could give false negative and positive results [9, 10, 11, 12, 13, 14]. On the other hand, Western blot is a sensitive assay but it can also give false negative results for patients with altered immune responses or different disease stages possibly caused by HIV-1 infection [5, 6, 15, 16]. Thus, the genetic-based HIV-1 diagnosis is the most needed method to overcome the limitation of serological diagnosis.

In this study, the evaluation of the RDBR for plasma samples which was compared to the method of conventional RT-PCR and commercial ELISA assays, has

Table 1. Comparison of results between conventional RT-PCR and the RDBR (**A**), and the RDBR and ELISA (**B**) assays

A	No. (%) of specimens					
	RDBR+, RT-PCR+	RDBR+, RT-PCR-	RDBR-, RT-PCR+	RDBR -, RT-PCR-	Total RT-PCR+	Total DB+
HIV-1	62(71.26)	3(3.45)	0(0)	22(25.29)	62(71.26)	65(74.71)
B	ELISA+, RDBR+	ELISA+, RDBR-	ELISA-, RDBR+	ELISA-, RDBR-	Total RDBR+	Total ELISA+
HIV-1	61(70.11)	9(10.34)	4(4.60)	13(14.94)	65(74.71)	70(80.46)

RDBR: RT-PCR dot blot hybridization based on radioisotope ^{32}P. +: positive. -: negative

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been performed. Comparison of the RDBR and the conventional RT-PCR showed that the RDBR was more sensitive than the conventional RT-PCR as can be seen from Table 1 in which the number of positive samples in the RDBR increases by 3.45%. The increase of the number of positive samples is caused by the ^{32}P that was immobilized on HIV-1 specific oligonucleotide. Several beacons can be attached on oligonucleotide such as ^{32}P, biotin, alkaline phosphatase, and sulphone. Among them, the ^{32}P yields the strongest signal that make the ^{32}P-based method to be the most sensitive [7]. If we consider the program in preventing the HIV-1 transmission, the percentage (3.45%) is very significant to hamper the HIV-1 transmission. Therefore, it is suggested that to use not only conventional RT-PCR assay for detection of HIV-1 but also a combined RT-PCR and dot-blot hybridization method. The combination method was also suggested by Lopez-Jimena and colleagues because it could increase the detection rate up to 90.62% [17].

The comparison of the RDBR and the ELISA showed conflicting results, because 9 RDBR negative samples were ELISA positive and 4 ELISA negative samples were RDBR positive (Table 1). Ly *et al*. has evaluated 7 HIV antigen-Ab combination assays, including the Vironostika kit, for detection of seroconversion, HIV Ab, and HIV antigen variants [18]. The result showed that of 50 HIV Ab positive samples, the Vironostika kit could detect only 44 of them. Moreover, Iqbal et al. reported only one false positive result by the Vironostika [19]. A report of the performance evaluation survey for HIV-1 Ab testing showed that of 964 HIV-1 positive samples, the Vironostika kit yielded only one false positive and 1 negative results [20]. A recent report showed that of 73 HIV-1 positive samples, sensitivity and specificity of the Vironostika kit were 100% and 97%, respectively [21]. Based on this data, the Vironostika kit could yield the false positive and negative results. The false negative results of ELISA tests could be caused by window-period phase and immunological dysfunction [5, 6, 15], while the false positive results could occur in people with acute non HIV-1 infection, recent influenza vaccination, autoimmune disorders, renal failure, cystic fibrosis, multiple pregnancies, liver disease, parenteral substance abuse, hemodialysis, and blood transfusions [22-24]. The researchers recommended using at least two or more ELISA kits to improve the sensitivity and specificity of assay [18, 19]. In this study, we only used one ELISA kit (the Vironostika kit); therefore, the ELISA results might not represent the actual test results.

The RDBR method is supposed to be a more high sensitive and specific method than the ELISA since the Vironostika kit still yields false positive and negative tests. Results of this experiment showed that 9 of 87 samples (10. 34%) and 4 of 87 samples (4.60%) were false positive and negative, respectively. Several researchers have reported the comparison studies of dot blot hybridization with the ELISA methods for detection of other microorganisms [25, 26, 27, 28]. They found that the PCR/RT-PCR-dot blot hybridization was much more sensitive and specific than the ELISA or serology assay. Another possible reason is that the primers used in this study are not able to amplify particular HIV-1 strains resulted from the high mutation rate of the HIV-1 genome. The assumptions need to be confirmed in the future study by using a high sensitive counterpart method such as real time RT-PCR.

CONCLUSIONS

The RDBR, a combined conventional RT-PCR and dot blot hybridization method was succesfully developed. It has high potential to be used as an alternative assay in screening large blood specimens particularly for surveillance studies. This method was more sensitive than conventional RT-PCR by showing the increasing number of positive specimens

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about 3.45%. In comparison with ELISA, the RDBR method showed lower sensitivity and specificity than ELISA ; however, the results need to be validated in the future because of the potentially false results that ELISA can produce, as having been reported by many researchers.

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