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ELISA versus Nested PCR for Detection of Hepatitis B Co-infection in HIV-infected People in Bandung Indonesia

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Abstract

The number of Human Immunodeficiency Virus infected patients in Bandung is increasing. HIV patients are often suffering from Hepatitis B Virusco-infection. The co-infection status must be detected using the accurate method. This study aims to compare the accuracy of Enzyme-Linked Immunosorbent Assay and nested Polymerase Chain Reaction in detecting HIV-HBV co-infection in Bandung. The research method used is experimental. The sample used in this study was 50 people. All samples had met the inclusion criteria. The presence of hepatitis B surface antigen in the serum was detected using a qualitative ELISA Wantai kit, while sHBsAg gene in the whole blood was amplified using 2 pairs of the specific primary. The results obtained from both methods were analyzed to determine sensitivity, specificity, and accuracy values. The result of Hepatitis B detection using ELISA method showed that 15 samples (30%) were positive, 1 sample (2%) was a false positive, and 3 samples (6%) were the false negative. Moreover, the nested PCR method showed that 18 samples (36%) were positive without false positive or false negative result. These results showed that ELISA has a sensitivity value of 83.33%, specificity of 96.87%, and the accuracy of 92%, while Nested PCR has a value of 100% for all three parameters. Therefore, it can be concluded that the Nested PCR method was more accurate than ELISA to detect Hepatitis B co-infection in HIV patients in Bandung.The Nested PCR can be recommended for detecting the other co-infection cases.

Keywords: accuracy, HBsAg, co-infection, sensitivity, specificity

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1. Introduction

Human Immunodeficiency Virus (HIV) is the major health problem worldwide including in Indonesia. The Indonesian Health Ministry (2018) reported that the cumulative number of HIV-infected people in Indonesia are 280,623 people with the majority in productive age. Bandung is one of the cities where the number of HIV-infected people increases significantly, with a prevalence of 17.34 % (Dinas Kesehatan Provinsi Jawa Barat, 2016). The high number of HIV cases in Bandung made the local-government issue the

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regulation of HIV prevention recorded in the Bandung City regulation Number 12 of 2015.

HIV is a virus that attacks the body's immune system so that infected people are susceptible to various pathogens, such as *Candida albicans*, Mycobacterium spp., and *Toxoplasma gondii*(Madigan, 2012). In addition, the HIV sufferers often has co-infection, one of which is the Hepatitis B Virus (HBV) (Kourtis & Jamieson, 2012; King & Hagemeister, 2016). HIV-HBV co-infection often occurs because of the transmission pathway similarity, as through blood, semen, and vaginal fluid (Naully & Romlah, 2018).

HIV-HBV coinfected patients need different treatment with HIV or HBV monoinfected patients (Owolabi, 2014). Chronic Hepatitis B in HIV-positive patients is 3 to 6 times more common than that in HIV-negative patients (Bratanata, Gani, & Karjadi, 2015). According to Ranjbar et al. (2011), HIV-HBV co-infection accelerate cirrhosis and hepatocellular carcinoma, also increase the HBV replication and HBV reactivation risk. Agyeman & Ofori-Asenso (2016) also reported that HIV-HBV co-infection could increase mortality by 36%. Therefore, Hepatitis B co-infection status in HIV-infected patients must be detected as early as possible using an accurate method.

Although there are several markers available that can be used to detect HBV, HBsAg is still the main marker because it appears in the early acute infection and disappears during recovery (Chameera, Noordeen, Pandithasundara, & Abeykoon, 2013). The presence of HBsAg in serum or plasma indicates an active infection of chronic or acute Hepatitis B (Khan et al., 2010). HBsAg can be detected by several methods, such as immunochromatography, Enzyme-Linked Immunosorbent Assay (ELISA), and Polymerase Chain Reaction (PCR) (Asaduzzaman, Milon, Juliana, Islam, & Kabir, 2018).

In the developing countries as Indonesia, the method commonly used to detect HBsAg is immunochromatography because it is cheaper, and does not require an expert and sophisticated laboratory equipment (Khan et al., 2010). However, several studies have shown that immunochromatography has lower sensitivity and specificity than ELISA (Chameera et al., 2013; Amin, 2019). Besides, there are also studies reporting that ELISA has lower detection performance than PCR, specifically double step PCR or nested PCR (Bolad, Hashim, & Lutfi, 2011; Amini et al., 2017). On the other side, Olotu et al. (2016) reported that the Nested PCR is vulnerable to contaminate and false positive, specifically at the first amplification. All of these studies compared the accuracy method for Hepatitis B screening in negative HIV sample. Until now, the study comparing the performance of the ELISA and nested PCR for the detection of HIV-HBV co-infection cases is rarely. Therefore, this study aimed to compare the accuracy of the ELISA and nested PCR in detecting hepatitis B co-infection in HIV-infected people in Bandung.

2. Methods

2.1. Samples

The number of samples used in this study was 50 people, with the inclusion criteria: people were HIV infected, had never done a Hepatitis B examination and domiciled in Bandung. All samples involved in this research knew the objectives, benefits, and procedures of the study and signed informed consent. Before the specimen collection, all subjects were asked to fill out alifestyle questionnaire created by researchers in 2019. The sample collection procedure has been approved by the Health Research Ethics Commission of Stikes Jenderal Achmad Yani with ethic number 08/KEPK/V/2019.

2.2. HBsAg Detection

The presence of HBsAg in the serum was detected using qualitative ELISA-sandwich method. The kit used was Wantai HBsAg ELISA. HBsAg was recognized by anti-HBs labeled Horseradish Peroxidase (HRP) enzyme. Then, the substrate used was Tetramethyl benzidine (TMB). The inspection results were declared valid after fulfilling the quality control requirements in the insert kit. Samples tested positive for HBsAg if the absorbance distribution of the sample with a cutoff value ≥ 1 (S/Co ≥ 1).

2.3. sHBsAg Gene Detection

The DNA genome from the whole blood was isolated using the Wizard® Genomic DNA Purification Kit. The concentration and purity of isolated DNA were measured using the nanodrop. Moreover, the HBsAg gene was recognized using 2 pairs of the specific primer. Then, the primer sequence was obtained from the research of Liu et al.(2010) (Table 1), but there were some modifications for the reaction composition and amplification conditions. The total volume of amplification reaction was 25 µl consisting 17.5 µl ddH2O; 2.5 µl buffer MgCl2 10X; 1 µl dNTPs 10 mM; 0.5 µl primer sHBsAg F1 10 mM; 0.5 µl primer sHBsAg R1 10 mM; 1 µl Taq DNA Polymerase 5U/µl; and 2 µl of sample with minimal DNA concentrations of 50 ng/µl. The DNA genome sample was used at the first amplification, and the first DNA-amplification result will be used in the second stage. In this study, the DNA genome from Hepatitis B-infected patient was used as a positive control, and the DNA genome from HIV or Hepatitis B negative patient was used as a negative control.

TABLE 1: Primer Sequence for sHBsAg Gene Amplification

Primer	DNASequence	Amplicon Size (bp)
Forward First-stage	5'-TCGTGTTACAGGCGGGGTTT-3'	513
Reverse First-stage	5'- CGAACCACTGAACAAATGGC-3'	
Forward Second-stage	5'- CAAGGTATGTTGCCCGTTTG-3'	233
Reverse Second-stage	5'- GGCACTAGTAAACTGAGCCA-3'	

TABLE 2: The Detection Results of Hepatitis B Co-infection in HIV-infected Patients in Bandung

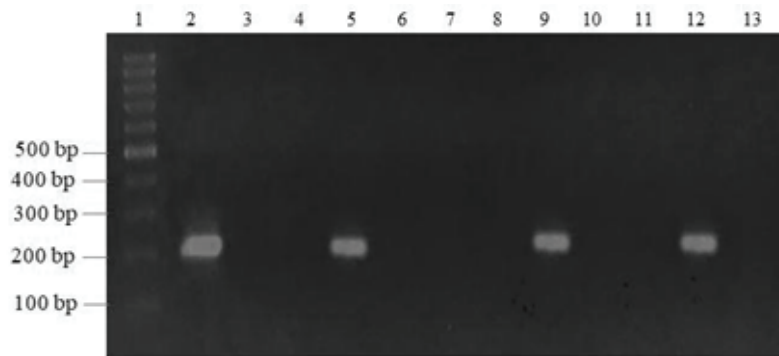| Detection Method	True Positive	True Negative	False Positive	False Negative
ELISA	15	31	1	3
Nested PCR	18	32	0	0

Figure 1: The electropherogram representation of the amplification result of the second-stage DNA HBV. Lane 1: Thermoscientific ladder 1 kb; Lane 2: Positive control; Lane 3: Negative control; Lane 4–13: Samples number 1–10.
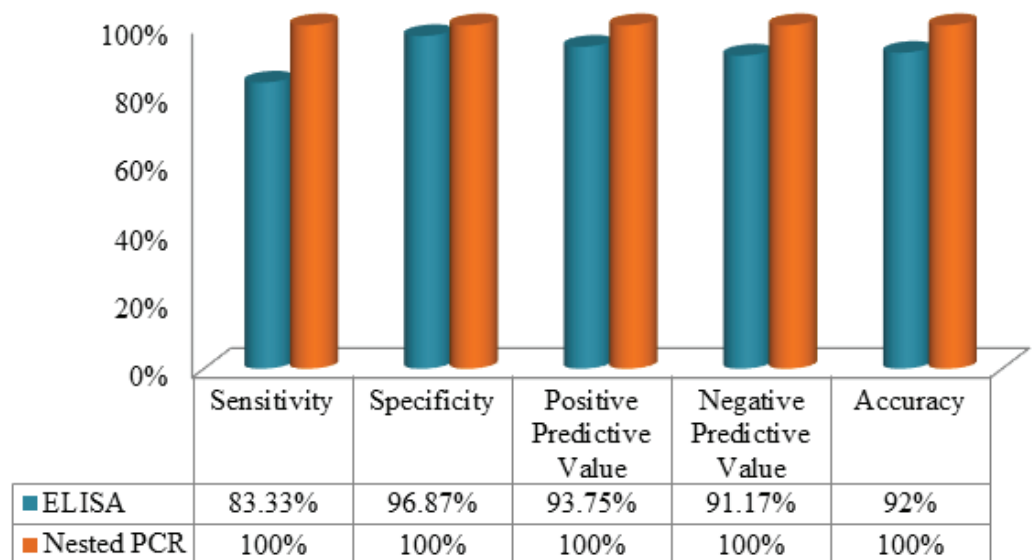

	Sensitivity	Specificity	Positive Predictive Value	Negative Predictive Value	Accuracy
ELISA	83.33%	96.87%	93.75%	91.17%	92%
Nested PCR	100%	100%	100%	100%	100%

Figure 2: The Comparison of ELISA and Nested PCR in detecting Hepatitis B Co-infection in HIV-infected Patients in Bandung.

The amplification process commenced with the DNA pre-denaturation for 5 minutes at 94°C. Then, the amplification process was performed by 35 cycles consisting of denaturation at 94°C for 20 seconds, annealing at 56°C for 30 seconds, and elongation at 72°C for 45 seconds. Furthermore, the final elongation was performed at 72°C for 10 minutes. Then, the amplification results were visualized using the electrophoresis with 1% agarose concentration.

2.4. Data Analysis

The results obtained from both methods were compared and analyzed to determine the value of sensitivity, specificity, and accuracy. The formula used is as follows (Wang, Zeng, & Zhu, 2010):

$$\text{Sensitivity} = \frac{\text{True Positive}}{\text{True Positive} + \text{False Negative}} \times 100\%$$

$$\text{Specificity} = \frac{\text{True Negative}}{\text{True Negative} + \text{False Postivie}} \times 100\%$$

$$\text{Accuracy} = \frac{\text{True Positive} + \text{True Negative}}{\text{True Positive} + \text{True Negative} + \text{False Postivie} + \text{False Negative}} \times 100\%$$

Besides the three parameters, the positive and negative predictive values of each method also were decided using the following formula:

$$\text{Positive Predictive Value} = \frac{\text{True Positive}}{\text{True Positive} + \text{False Positive}} \times 100\%$$

$$\text{Negative Predictive Value} = \frac{\text{True Negative}}{\text{True Negative} + \text{False Negative}} \times 100\%$$

3. Results

Hepatitis B Co-infection in this study was detected using ELISA and nested PCR on 50 HIV-infected people, consisted of 35 men (70%) and 15 women (30%), aged 19-58 years old with majority senior high-school educated. Some of the samples were students, housewives, and former prisoners. Moreover, the HBsAg detection result using ELISA showed that 15 samples (30%) were positive. The result was valid because it has met the quality control kit requirements used: the absorbance blank value at < 0.008; negative control at ≤ 1; and positive control at ≥ 0.8.

The detection of sHBsAg gene at the first stage showed that the 513 bp sHBsAg gene was successfully amplified from 18 samples (result not shown). The sHBsAg gene size was according to the pre-designed amplicons (Table 1). The second stage amplification electropherogram also showed the DNA band formed from 18 samples (Figure 1). The DNA band size was 233 bp, parallel to the DNA band on the positive control. The sHBsAg

gene amplification process proved to be specific because there was not shown DNA band or smear formed on negative control (Figure 1, Lane 3).

After compared, there were some differences in the detection results between ELISA and Nested PCR (Table 2). There were 3 negative samples (6%) in ELISA, but positive in the Nested PCR. Besides, there was also 1 sample (2%) that was positive in ELISA, but negative in the Nested PCR. Furthermore, the laboratory results obtained were compared with the clinical symptoms of samples. The result showed that 3 samples which tested negative had Hepatitis B symptoms. Then, 1 sample that tested positive did not feel clinical symptoms of Hepatitis B, even claimed to get the Hepatitis B vaccine four years ago.

These results showed the sensitivity and specificity differences affecting the accuracy of both methods (Figure 2). The false-negative and false-positive results in ELISA showed that the sensitivity, specificity, and accuracy of the method were lower than those of Nested PCR. Besides, ELISA also had a positive predictive value (PPV) and a lower negative predictive value (NPV).

4. Discussion

The accuracy of a method detecting the infection is influenced by the sensitivity and specificity values. Sensitivity is the method ability to give a positive result for someone truly infected, while specificity is the method ability to give the negative result to someone truly not infected. According to Hayder et al. (2012), the method with the high levels of sensitivity and specificity are required to detect the infection to minimize the false-positive and false-negative results. Beside of sensitivity and specificity values, the choice of detection method is also influenced by the PPV and NPV. PPV is the probability of someone infected when getting a positive test result, while the NPV is the probability of someone not being infected when getting the negative test result. According to Asaduzzaman et al. (2018), the high sensitivity and negative predictive values are more important parameters than the specificity and high positive predictive values in selecting detection methods.

Both methods used in this research have successfully proven the existence of Hepatitis B co-infection cases in HIV-infected people in Bandung. Although the study of HIV-HBV co-infection in Bandung is rarely, these results were in line with several studies conducted in other cities. HIV-HBV co-infection were reported in Semarang and Surakarta, at 19.7% (Sepsatya, 2011) and 3.9% (Ew & Prasetyo, 2015) respectively. Based on the questionnaire data in this study, it was known that HIV-HBV co-infection

cases in Bandung were influenced by unsafe sexual activity, injection-drug use, and vaccination history.

Although both methods can detect the HBV in the specimen, this study proved that the sensitivity value of ELISA (83.33%) was lower than that of nested PCR (100%). Previous studies have also proven that the sensitivity of ELISA was not as good as that of molecular methods such as single-step PCR (conventional PCR) and nested PCR. Bolad et al. (2011) reported that the sensitivity value of ELISA was much lower than the conventional PCR in the detection of Hepatitis B infection in the blood of healthy donors. In that study, ELISA did not show the false-positive result, but the number of false-negative results was 34.8%. Kurdi et al. (2014) also successfully proved that many false-negative results were found in ELISA indicating that the method was less sensitive than the Nested PCR. Bratanata (2015) reported that the sensitivity of nested PCR achieved 10^2 genome copies per milliliter of serum. Moreover, Liu et al. (2010) reported that 20 copies of the gene per ml of serum could still be detected using the Nested PCR. The level of PCR sensitivity can be increased by conducting a hybridization analysis using a DNA tracker labeled radioisotope. The detection ability increased to 105 times higher than that of conventional PCR process (Bolad et al., 2011).

Beside of the sensitivity, this study also proved that the specificity of ELISA was lower than that of the Nested PCR. This result was in line with Nie et al. (2012) and Hudu et al. (2016) reporting that the Nested PCR was more specific than ELISA and conventional PCR. The non-specific detection result of ELISA is usually caused by the cross-reaction between the antibody detection used. The majority of PCR detection methods are specific because the primer has been specifically designed to recognize the sHBsAg gene sequence in HBV. A researcher generally will test the specificity of the primary in the NCBI program before use. Although Olotu et al. (2016) reported that the Nested PCR was vulnerable to contaminate and false positive, this was not proven in this study. The contamination and false positive can be minimized by the sterile work technique, the primary sequence selection, and the prover amplification condition.

The accuracy of the nested PCR (100%) also has been proven better than that of ELISA (92%). This result is supported by Amini et al. (2017) that reported that from the 40 samples, the accuracy of ELISA was lower than that of PCR specifically to detect Hepatitis B co-infection in HIV-infected people. Moreover, HIV-infected people often have occult Hepatitis B infection, a condition when HBV DNA can be detected in serum or liver tissue but HBsAg is not detected (Liu et al., 2010; Bratanata et al., 2015; Olotu et al., 2016). According to Honge et al. (2014), in the HIV-HBV co-infection case, most

of the samples with the false-negative result commonly had lower HBsAg concentration and HBV DNA than the positive, because HIV-infected patients were generally getting the Anti-Retroviral Therapy. HIV Reverse Transcriptase Inhibitor contained in the treatment which can fight the HBV actively so reducing the HBV replication and HBsAg concentration (Heathcote et al., 2011). Besides, it is also possible that someone with HIV-infected has the HBV and Hepatitis C Virus (HCV) co-infection simultaneously. There are so many cases widely reported, such as reported in Zhou et al. (2012) and Saraswati et al.(2015). The presence of HCV core protein can inhibit the HBV replication, so the amount of HBsAg produced was very low (Bratanata et al., 2015).

Besides the lower HBsAg concentration, the false-negative result can be caused by the genetic mutation in HBV. Taffon et al. (2014)proved that HBV in HIV-infected patient the genetic mutation, especially gen preS dan S so HBsAg protein undergoes the structural changes. It is supported by Sadeghi et al. (2017) reporting that ART use as lamivudine can encourage the emergence of the surface gene variant that cannot be detected by the immunological method as ICT or ELISA. Mutation in the determinant antigenic "a" region from HBsAg can change HBsAg protein conformation that was difficult to recognize by commonly used antibodies (Liu et al., 2010). It leads to the decline of diagnostic accuracy. Olotu et al. (2016) stated that the most accurate method for detecting HBV in HIV-infected patients was the molecular-based method.

5. Conclusion

Based on the study, it can be concluded that the Nested PCR is more accurate than ELISA for detecting Hepatitis B co-infection in the HIV-infected people in Bandung. The value of sensitivity, specificity, and accuracy of the method was 100%. The Nested PCR can be recommended for detecting the other co-infection cases.

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