

DOI:

10.22301/IJHMCR.2528-3189.568

Article can be accessed online on:

<http://www.ijhmcr.com>

ORIGINAL ARTICLE

**INTERNATIONAL JOURNAL
OF HEALTH MEDICINE AND
CURRENT RESEARCH**

**PERFORMANCE EVALUATION OF THE AFIAS-6 ANTI-HCV
ASSAY FOR THE DETECTION OF HEPATITIS C
VIRUS ANTIBODIES**

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ARTICLE INFO

Article History:

Received 20th July, 2017

Received in revised form

07th Agustus, 2017

Accepted 22th Agustus, 2017

Published online 28th September,
2017

Key words:

Hepatitis C, Hepatitis C antibodies,
sandwich immunoassay.

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ABSTRACT

Background: The accurate examination for diagnosis of HCV infection is very important in order to receive an adequate treatment. The serology test which detects specific antibodies to HCV is recommended. The AFIAS-6 anti-HCV assay was recently introduced in Indonesia. This study aimed to evaluate performance of the AFIAS-6 anti-HCV in comparison to Cobas e601, which is routinely used in Dr. Cipto Mangunkusumo General Hospital, for the detection of anti-HCV antibodies in the population of Indonesia.

Methods: 200 samples, which comprised of 100 positive samples and 100 negative samples according to the Cobas e601, were tested by AFIAS-6. The Cohen's Kappa coefficient was used to assess the agreement.

Results: The Kappa coefficient result was 0.890 ($p=0.032$, 95%CI 0.827-0.953) and total agreement rate between AFIAS-6 and Cobas e601 anti-HCV assays was 94%. The sensitivity and the specificity of the AFIAS-6 assay were calculated to be 93% and 95%, respectively.

Conclusions : The AFIAS-6 anti-HCV assay showed good agreement with the Cobas e601 anti-HCV assay. The result indicates that AFIAS-6 is a reliable tool for detection of hepatitis C virus antibody, and may be useful in small or medium sized laboratories or as a back-up analyzer in bigger ones.

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Citation: Diana Shintawati Purwanto¹, Yusra^{1,2}, 2017 "Performance Evaluation Of The AfiAS-6 Anti-Hcv Assay For The Detection Of Hepatitis C Virus Antibodies", *International Journal of Health Medicine and Current Research*, 2, (03), 568-572.

INTRODUCTION

Hepatitis C is an infection of the liver tissue caused by hepatitis C virus (HCV). Currently hepatitis is a global health problem. It is estimated that approximately 130-170 million people (2-3% of the world's population) suffer from hepatitis C infection. Hepatitis C, especially in its chronic form, is associated with high morbidity and mortality. Each year more than 350,000 deaths are associated with HCV infection, mostly due to liver cirrhosis and hepatocellular carcinoma (HCC). An estimated 27% of cirrhosis and 25% of HCC are associated with hepatitis C worldwide, and the prevalence of this disease is greater in countries with high infection burden. For example, HCV causes up to 90% of HCC cases in Japan [1]. In Indonesia, based on surveillance data from the Directorate General of Disease Control and Environmental Health, Ministry of Health of the Republic of Indonesia, positive anti-HCV were obtained at 35,453 samples (0.7%) in 2007-2012 [2].

The accurate examination for diagnosis of HCV infection is very important, so that an adequate treatment can be carried out. The diagnosis of HCV is based on two types of tests, the serology test which detects specific antibodies and the molecular test that detects HCV-RNA. The detection of HCV antibodies is generally performed by using ELISA technique immunoassay (EIA) and chemiluminescence immunoassay (CLIA). Three different generations of anti-HCV test kits have been developed to date. The first generation HCV EIA detected only antibodies against the non-structural region 4 (NS4) with recombinant antigen c100-3. The second generation added NS4 region, and the third generation used today includes NS5 region and a reconfiguration of the core and NS3 antigens. The sensitivity and specificity have been markedly improved across three generations [3,4].

This study aimed to evaluate performance of the AFIAS anti-HCV on AFIAS-6 analyzer (Boditech Med Incorporated, Chuncheon, Republic of Korea) in comparison to the Elecsys anti-HCV II assay on Cobas e601 analyzer (Roche Diagnostics, Mannheim, Germany), for the detection of anti-HCV antibodies in the population of Indonesia, especially Cipto Mangunkusumo hospital. The AFIAS-6 anti-HCV assay was recently introduced in Indonesia. Currently, Cobas e601 is routinely used in Cipto Mangunkusumo hospital, as the national referral hospital center, for the detection of anti-HCV antibodies.

METHODS

During August-September 2016, samples underwent anti-HCV testing at Cipto Mangunkusumo hospital were collected consecutively until 200 samples achieved, which comprised of 100 positive samples and 100 negative samples according to the electrochemiluminescence immunoassay (ECLIA) by Cobas e601. These sera were then stored frozen at -20°C until assayed by using AFIAS-6 in September 2016. All sera were thawed once and allowed to stand at the room temperature before examination. Hemolysed, lipemic, and icteric samples were excluded. The two anti-HCV assays were carried out according to the manufacturers' instructions.

The Cobas e601 uses ECLIA method. In this method, sample is incubated with two specific antigens, biotinylated HCV-specific antigens and HCV-specific antigens labeled with a ruthenium complex react to form a sandwich complex. After the addition of streptavidin-coated microparticles, the complex binds to the solid phase via interaction of biotin and streptavidin. The reaction mixture is aspirated into the measuring cell where the microparticles are magnetically captured onto the surface of the electrode. Application of a voltage to the electrode then induces chemiluminescent emission which is measured by a photomultiplier. The analyzer automatically calculates the cutoff based on the measurement of A-HCV II Cal1 and A-HCV II Cal2. The result of a sample is given either as reactive or non-reactive in the form of a cutoff-index (signal sample/cutoff) by comparing the electrochemiluminescence signal obtained from the reaction product of the sample with the signal of the cutoff value. The interpretation of the results: samples with a cutoff-index (COI) < 0.9 are non reactive, COI > 0.9 and < 1.0 are considered borderline, and COI > 1.0 are reactive [5]. Nonreactive result is considered there are no antibodies against HCV, and concluded as negative. Reactive result is considered there are antibodies against HCV, and concluded as positive.

The AFIAS-6 uses sandwich immunodetection method. Cartridge has a detection buffer containing dried recombinant HCV antigens and anti-chicken IgY, which are both labeled with fluorescence. When mixing with the sample, recombinant HCV antigens bind to the antibodies in the sample, forming antigen-antibody complex. When it migrates onto nitrocellulose matrix, antibodies in the sample are captured by the other recombinant HCV antigens immobilized on the test

strip, to form a sandwich complex. In line with that, the fluorescence labeled anti-chicken IgY binds to the chicken IgY fixed to the control strip. Detectors quantify analytes by measuring the fluorescence on the test strip induced by laser. The intensity of fluorescence formed is proportional to the concentration of the antibodies contained in the sample. The result of the samples is given as positive or negative or indeterminate, and in the form of COI. The interpretation of the results: samples with $COI \leq 0,9$ is negative, $COI > 0.9$ to <1.0 is indeterminate, and $COI \geq 1,0$ is positive [6].

In this study, Cohen's Kappa coefficient was used to assess the agreement, by using Statistical Product and Service Solution (SPSS) ver.22. The ethical review was obtained from the health research ethics committees of Faculty of Medicine the University of Indonesia.

RESULTS

The precision of AFIAS-6 Anti-HCV was evaluated by using the negative and positive control materials recommended by the manufacturer, and one indeterminate sample. The intra-assay precision for negatif and positive controls was evaluated in 10 replicates within one run. The intra-assay precision for sample was evaluated in 5 replicates within one run. The inter-assay precision was evaluated by using the negatif and positive control materials and run once a day for 10 days consecutively. Precision results for AFIAS-6 are shown in Table 1.

Table 1. Intra-assay and inter-assay precisions of AFIAS-6.

Control and sample	Intra-assay			Inter-assay	
	Negative	Positive	Sample	Negative	Positive
N	10	10	5	10	10
Target value	0.00	4.50	-	0.00	4.50
Mean	0.06	4.15	1.79	0.04	4.47
SD	0.10	0.30	0.22	0.04	0.26
%CV	1.85	0.07	0.12	1.1	0.06
% d	-	-7.8 – 6.9	-	-	-0.5 - 7

From 200 samples (100 positive and 100 negative) based on the Cobas e601 anti-HCV assay, 97 samples were positive, 1 sample indeterminate, and 102 negative according to the AFIAS-6. The results were concordant in 188 samples but discrepant in 12 samples,

between the two analyzers. There were 4 samples negative by Cobas e601 but positive by AFIAS-6, 1 sample negative by Cobas e601 but indeterminate by AFIAS-6, and 7 samples were positive by Cobas e601 but negative by AFIAS-6. These data are summarized in Table 2. Results of the anti-HCV examination in 200 samples were grouped into three categories: positive, indeterminate, and negative.

The Kappa coefficient result was 0.890 ($p=0.032$, 95%CI 0.827-0.953) and total agreement rate was 94%. Defining the Cobas e601 assay as “reference test”, the sensitivity and the specificity of the AFIAS-6 assay were calculated to be 93% and 95%, respectively.

Table 2. Correlation between the results of the Cobas e601 and AFIAS-6 anti-HCV assays.

AFIAS-6 anti-HCV assay	Cobas e601 anti-HCV assay			Total
	Positive	Indeterminate	Negative	
Positive	93	0	4	97
Indeterminate	0	0	1	1
Negative	7	0	95	102
Total	100	0	100	200

DISCUSSION

In this study, for preliminary examination the AFIAS-6 anti-HCV assay showed good reproducibility with intra-assay and inter-assay precisions were less than 2%. Two hundred samples were used consisting of 100 positive samples and 100 negative samples based on the results of anti-HCV from Roche Cobas®e601. Based on the sample size calculation from Kappa Cohen formula, the minimum sample size required to this comparison of two tests was 144, and so 200 samples were considered have fulfilled the required sample size for this study.

Test results from Cobas e601 and AFIAS-6, of the 200 samples, 188 samples were in agreement (94%), consisting of 93 samples were positive and 95 samples were negative, and 12 samples were discrepant (6%). Results of this study are comparable to other studies that have agreement ranging from 94.5 to 99.5% [7,8]. The Kappa coefficient result was 0.890 ($p=0.032$, 95%CI 0.827-0.953). The interpretation of Kappa value was very good in accordance with the interpretation put forward by Landis and Koch (Kappa 0.81-1.00 means almost perfect agreement) [9].

For the sensitivity and specificity, a recent study of the AFIAS-6 system showed 93% sensitivity and 95% specificity. The results are similar to those reported in other studies, in which the sensitivity and spesificity

values were ranging from 86.8 to 100% and from 96.5 to 99.9%, respectively [7,8,10].

There were 12 samples (6%) exhibited discordant results between the two tests evaluated in this study. This may be due to differences in antigens or epitopes or differences in labeling substances for signal detection in the assay. The Cobas e601 test is a two step immunoassay based on the ECLIA and employs Core, NS3, and NS4 peptides as antigens, on the other hand AFIAS-6 is a sandwich immunodetection method with final fluorescence detection and uses Core, NS3, NS4, and NS5 peptides as antigens. Generation I anti-HCV used recombinant antigens containing epitopes from the NS4 region (c100-3) HCV genome. Generation I had low sensitivity to the population with a high prevalence ($\pm 80\%$) and lead to a high false positive (up 70%) in the group of blood donors. Generation II used recombinant antigens from the core region (c22-3), NS3 (c33c), and NS4 (part of c100-3). Generation II increases the sensitivity to $\pm 90\%$ and $\pm 99\%$ specificity. Generation III EIA has been equipped with NS5 epitopes to increase the sensitivity and specificity to $> 99\%$ [11,12].

There were 4 samples negative by Cobas e601 but tested positive by AFIAS-6. Three of these discrepant samples showed very weak reactivity in the AFIAS-6 assay (COI 1.06-3.35), and one sample had COI 10.77. The Centers for Disease Control and Prevention (CDC) recommends additional tests on the results of screening tests. Supplemental testing, recombinant immunoblot antibody assay (RIBA) or nucleic acid test (NAT), may be limited to screening test-positive patients with average COI < 3.8 , as anti-HCV positive samples with average COI ≥ 3.8 would be highly predictive of the RIBA positivity ($\geq 95\%$). RIBA and other immunoblot assays are commonly used to confirm a reactive result at an anti-HCV screening test. The same antigens as in EIAs are used in these assays, but the antigens are separately coated on a membrane and the result depends on the number of bands present on the membrane. The immunoblot assays, more specific than EIAs, can confirm a true positive anti-HCV result, however the disadvantage is unable to confirm an active HCV infection. On the other hand, HCV-RNA molecular test can confirm the presence of HCV infection. In addition, HCV-RNA tests can explain the patients with clinically suspected infection with hepatitis C but the results of anti-HCV EIA test is dubious. For example immunocompromised patients whose screening results are negative for anti-HCV due to antibodies are not produce enough, or in patients with acute hepatitis C

negative for anti-HCV whose antibodies appear after one month of the acute phase [13,14].

Another sample was indeterminate according to AFIAS-6 (COI 0.94), but negative to Cobas e601 (COI 0.094). An indeterminate anti-HCV result indicates that the antibody status cannot be determined. It can indicate a false-positive anti-HCV screening test result (the most likely interpretation among those at low risk for HCV infection), or can occur as a transient finding in a recently infected person who is in the process of seroconversion, or can be a persistent finding among persons chronically infected with HCV. If NAT is not performed, another sample should be collected for repeat anti-HCV testing (≥ 1 month later) [14].

There were seven positive samples by Cobas e601 but tested negative by AFIAS-6. These samples showed positive results with COI between 10.24 and 95.30. The COI of these results are ≥ 3.8 , and so according to the CDC would be highly predictive of the RIBA positivity ($\geq 95\%$). The cause of this difference is most likely due to differences in the epitopes used by both methods despite the same antigenic region.

Anti-HCV negative result indicates the absence of detectable antibodies to HCV. False negative can occur in the past HCV infection and possible samples have anti-HCV antibody levels below the limit of detection, or the patient in the process of seroconversion (window period between 45-68 days). A positive result indicates three possibilities : HCV infection is currently active, past HCV infection which has completed, or false positives [3].

In this study all discrepant samples were investigated by medical record review. The medical record review involved the examination of previous laboratory results as evidence of chronic HCV infection or the presence of other clinical conditions that could interfere with the accuracy of results such as autoimmune disease, pregnancy, rheumatoid factor (RF), antinuclear antibody (ANA), the infection of cytomegalovirus (CMV), Epstein-Barr (EBV), hepatitis A virus (HAV), hepatitis B virus (HBV), herpes simplex virus (HSV), and Syphilis. Among 12 samples, there was one negative sample on Cobas e601 but positive by AFIAS-6 (COI 1.96) had a positive ANA result. The one negative sample by Cobas e601 (COI 0.094) but indeterminate by AFIAS-6 (COI 0.94) had a reactive result to hepatitis B surface antigen (HBsAg). Likewise, one positive samples by Cobas e601 (COI 28.13) but negative by AFIAS-6 had a positive RF result. Positive results in the first two samples above with low COI

could be a false positive because of cross-reactions with other antibodies.

From the aspect of tools and work procedures, AFIAS-6 works automatically, and so the error of mixing reagents can be minimized. The reaction time and sample volume required are 12 minutes and 100 μ L, respectively. In addition, the AFIAS-6 analyzer uses a single dose and ready-to-use reagent, is light and simple to operate [6].

In conclusion, based on Kappa result, the AFIAS-6 anti-HCV assay showed very good agreement with the Cobas e601 anti-HCV assay. These results indicate that AFIAS-6 is a reliable tool for detection of hepatitis C virus antibody, and it may be useful for small-to medium sized laboratories or as a back-up analyzer in bigger ones. Nonetheless, discordant samples need to be followed up with confirmatory assay.

Authors' Disclosures of Potential Conflicts of Interest

The authors had no conflicts of interest to declare in relation to this article.

Acknowledgments

The authors are grateful to Boditech Med Inc. and PT. Tripatiria Andalan Medika for providing the reagents.

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