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RELIABILITY OF AMYLOID DETECTION IMMUNOASSAY KITS FOR ALZHEIMER'S DISEASE SCREENING: A PRELIMINARY STUDY

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ABSTRACT

This study aimed to evaluate the reliability of the amyloid detection kit which was developed as an alternative screening test for Alzheimer's disease compared to commercially available kits. The samples used in this study were sera samples and cerebrospinal fluid from adult long-tailed macaques (*Macaca fascicularis*). This study used 3 commercial Enzyme-Linked Immunosorbent Assay (ELISA) kits and in-house ELISA kit based on a monoclonal amyloid beta 42 antibody to detect the presence of amyloid beta 42 protein in the serum and cerebrospinal fluid (CSF) of *Macaca fascicularis*. The validity test was carried out by comparing this in-house amyloid ELISA kit with similar commercial kits using the same serum and cerebrospinal fluid samples. Based on the results and interpretation of inter and intra-assay coefficients, the in-house ELISA kit has comparable amyloid β 42 detection to the commercially available ELISA kit.

Key words: Alzheimer's disease, amyloid β 42, ELISA, reliability kit, screening

ABSTRAK

Penelitian ini bertujuan melakukan kajian reliabilitas kit deteksi amiloid yang dikembangkan menjadi uji alternatif penapisan penyakit Alzheimer dibandingkan dengan kit komersial. Penelitian ini menggunakan 3 kit Enzyme-Linked Immunosorbent Assay (ELISA) komersial dan kit ELISA internal berbasis antibodi monoklonal amiloid β 42 untuk mendeteksi keberadaan protein amiloid beta 42 pada serum dan cairan serebrospinal (CSF) M. fascicularis. Penapisan berbasis pendeteksian dengan teknik ELISA dapat bekerja secara efektif dan akurat dalam mengoptimalkan bahan antibodi spesifik atau antibodi monoklonal terhadap peptida amiloid β 42. Berdasarkan hasil dan interpretasi dari nilai koefisien inter dan intra-assay, kit ELISA yang dikembangkan secara in house memiliki kemampuan deteksi amiloid β 42 yang menyamai Kit ELISA komersial yang telah tersedia.

Kata kunci: penyakit Alzheimer, amiloid β 42, ELISA, reliabilitas kit, penapisan

INTRODUCTION

Dementia is a general term used to describe various symptoms of cognitive dysfunction in the form of memory, emotion, decision making and other brain functions that interfere with daily activities (ALZI 2019). Dementia also known as major neurocognitive disorders that are not one specific disease but a group symptom that occur due to a disease.

Alzheimer's disease is the most common neurodegenerative disease and the cause of dementia (Sadigh-Eteghad *et al*. 2015). Alzheimer Indonesia (ALZI 2019) confirmed that as much as 60-70% of dementia is Alzheimer's disease. The prevalence of dementia tends to increase with increasing life expectancy. An increase in dementia prevalence has led to an increase in the number of people with Alzheimer's disease (Purba 2011). Alzheimer's disease gradually damages the nerves of the brain and generally occurs in the elderly, characterized by a decrease in memory, language, problem-solving, and other cognitive skills that can affect the ability to carry out daily activities. The decrease in cognitive function occurs because of nerve cells (neurons) in the part of the brain involved in cognitive function. Alzheimer's disease can even cause death to sufferers by causing malnutrition, brain death, and multiple organ failure due to the number of nerve cells that have died (Brookmeyer *et al*. 2007). The number of sufferers of Alzheimer's disease reached one million people in 2016 and this number is expected to double in 2030 and quadruple in 2050 with an increase in average life expectancy and the number of elderly (Kemenkes 2016; Kemenkes 2019).

Manufacturing a diagnostic tool for Alzheimer's disease in humans is a strategic effort that can be carried out to support the development of amyloid-beta (Aβ) 42 peptide detection through screening tests based on immunoassay techniques (Enzyme-Linked Immunosorbent Assay/ELISA) against markers which are early markers of Alzheimer's disease using specific antibodies or monoclonal antibodies against Aβ 42 peptides. The detection of this peptide is the first step in screening for Alzheimer's disease. So far, commercial ELISA kits have been imported, so they are difficult to reach and take a long time to acquire, so they have high commercial value.

Enzyme-Linked Immunosorbent Assay, one of the Enzyme Immunoassays (EIA), is a biochemical test that uses the basic concept of antigen binding to its specific antibody taking into account the color change to detect the presence of antigens such as proteins, peptides, hormones or antibodies in the sample fluid (Gan and Patel 2013). The principle of the ELISA test

is the use of an enzyme that is bound to the conjugate (secondary antibody) which reacts with the substrate causing a color change due to the presence of homologous antigen and antibody bonds (Wibawan and Soejoedono 2013). ELISA can be used as a screening test because it has a fairly high sensitivity value (Sumita *et al*. 2016).

Amyloid-beta belongs to the group of endogenous proteins and is secreted as a metabolic product of nerve cells. Physiologically, Aβ plays a role in ensuring brain function in transferring information between synaptic nerve cells, for example in learning and memory processes. This occurs because Aβ secretion can increase synapse activity (Abramov *et al*. 2009). In healthy people, the level of Aβ secretion is regulated through a feedback process, whereas in Alzheimer's sufferers there is a possibility that no feedback reaction occurs so that Aβ production goes without any inhibition which causes buildup as amyloid plaques. Amyloid plaque is one of the causes of death of nerve cells (Purba 2011).

Animal models are animals that can provide a specific response that resembles the description of human disease. The type of animal model is choosed after many considerations in order to achieve relevant results (Sajuthi *et al*. 2016). The long-tailed monkey *(Macaca fascicularis)* was used as an animal model because the Aβ 42 peptide in this primate has similarities in structure and physiological function to the Aβ 42 peptide in humans. Several behavioral similarities and markers of Alzheimer's disease have been found in aged long-tailed monkeys with humans with Alzheimer's disease, making long-tailed monkeys as a potential model for future research on Alzheimer's disease (Darusman *et al*. 2013).

The aim of this study is to analyze the reliability of the amyloid detection kit which was developed as an alternative test for Alzheimer's disease screening compared to commercial kits and is expected to provide benefits in the form of information for determining preferences for researchers and health workers in choosing the kit.

MATERIALS AND METHODS

The research was conducted from January 2022-December 2022 at Microbiology and Immunology Laboratory, Primate Research Center, Institute for Research and Community Services, IPB University (PSSP-IPB).

Sample Collection

The samples used in this study were sera samples and cerebrospinal fluid from adult long-tailed macaques (*M. fascicularis*) as archived samples that have been kept frozen at the Microbiology and Immunology Laboratory of PSSP IPB from previous field research or surveillance. The blood collection procedure followed the PSSP LPPM IPB animal welfare code of ethics with the ACUC protocol approval number no. IPB PRC-18-B003.

Detection of Amyloid Protein

The presence of amyloid protein was detected using 3 commercial ELISA Kits using a microplate pre-coated with a monoclonal antibody against Aβ 42. A total of 100 µL of amyloid standard and serum sample were added, incubated for two hours at 37° C. The contents of the plate were discarded and 100 µL of detection reagent was added and incubated for one hour at 37° C. The plate was washed using 300 µL of washing solution, then added 100 µL of detection reagent followed by incubation for one hour at 37° C. After the plates were washed, the substrate solution was added and incubated for 10-20 minutes in a dark room at 37° C. The reaction was stopped by adding 50 µL of reaction-stopping solution and then the color formed was read using a microplate reader (Biorad, iMark) with a wavelength of 450 nm.

The presence of amyloid protein was detected using the in-house ELISA Kit using monoclonal antibodies against Aβ 42 which was developed as a coating antibody in the in-house ELISA diagnostic kit (antigen capture). The Aβ 42 monoclonal antibody mixed with sodium dodecyl sulfate was dissolved in a carbonate-bicarbonate buffer solution, put into an ELISA microplate and incubated for four hours at 37° C, then washed four times with 0.05% PBS-tween. The blocking agent was added to the microplate. After incubation, the plates were washed and serum added, followed by incubation for 1 hour at 37° C. Washing was repeated 4 times, then 100 µL of anti-monkey IgG peroxidase conjugate (1:10.000) was added to all wells and incubated for one hour at 37° C. Tetramethylbenzidine as a chromogen was added 100 µL to each well and incubated in the dark at room temperature for 20 minutes. The reaction was stopped by adding 50 µL of 2N sulfuric acid to each well and then the color formed was read using a microplate reader (Biorad, iMark) with a wavelength of 450 nm.

Data Analysis

Conformity between kits A and B was determined using calculation of the coefficient of variability and the validity test of the in-house amyloid kit ELISA results. The validity test was carried out by comparing this in-house amyloid ELISA kit with similar commercial kits that have so far been imported using the same serum and cerebrospinal fluid samples. The calculation of the coefficient of variability (CV) was the standard deviation of a set of measurements divided by the average of that set. The inter-assay CV is acceptable if the value is <15% and the intra-assay CV is acceptable if the value is <10%. Determination of the value of the inter-assay variability coefficient was obtained by the following calculation: %CV inter-assay is the standard deviation of plate optical density (SD value) divided by the mean of mean plate OD and multiple by 100%. The value of intra-assay variability was determined by a mean of % CV multiple by 100%.

RESULTS AND DISCUSSION

ELISA Test

This study used 3 commercial ELISA kits and an in-house ELISA kit based on a monoclonal Aβ 42 antibody to detect the presence of Aβ 42 protein in the serum and cerebrospinal fluid (CSF) of *M. fascicularis*. The optical density value of the ELISA Aβ 42 is presented in Table 1.

As shown in Table 1, the optical density value of the ELISA Aβ 42 using two commercial kits (kit B and C was very high, whereas, in the commercial ELISA kit A, the result was very low. This might be due to the Kit A was performed two weeks after the other two kits using the cold CSF samples, wherein CSF samples were known to have relatively low stability in storage (Teunissen *et al*. 2009). The volume of the CSF sample obtained was insufficient to be divided into several tubes before the CSF sample was frozen at -80° C. According to the consensus protocol for storing serum and CSF samples, blood collection should be performed at the same time as CSF sampling for biomarker testing. Then the samples were divided into several vials and stored in the freezer -80° C. Frozen samples were tested using kit B and C samples which were previously frozen at -80° C, then the remaining CSF samples were stored at cold temperatures (4-8° C) to continue testing using kit A.

In analysis of *M. fascicularis* serum samples, commercial kit B obtained the highest optical density value, followed by kit C and kit A. However, the optical density values of the serum sample were below the optical density values of the CSF sample. This was probably due to the inability of commercial ELISA kits to detect the presence of Aβ 42 protein. These commercial kits can only be used to detect the presence of amyloid protein from CSF samples.

The coefficient of intra-assay variability is a level of accuracy that can be known by repeated measurements when all external conditions are kept the same, both in terms of the sample, the day of the test, and the laboratory personnel who perform the test so that an overview of the variations between test replications in the same test can be known (Ramljak *et al*. 2013).

Table 1. The optical density value of the ELISA amyloid beta 42

Sample type	Commercial ELISA Kit A	Commercial ELISA Kit B	Commercial ELISA Kit C	*in-house* ELISA Kit
CSF Mf 1	0.007	1.310	1.660	0.121
Serum Mf 1	0.004	0.039	0.013	0.220

Table 2. Intra-assay value of amyloid beta 42 in-house ELISA kit

Sample ID	OD 1	OD 2	Mean OD	SD	%CV
8124	0.670	0.685	0.678	0.011	1.566
5570	1.563	1.496	1.530	0.047	3.097
3630	0.795	0.808	0.802	0.009	1.147
9373	0.038	0.059	0.049	0.015	30.617
3558	0.298	0.354	0.326	0.040	12.147
4030	0.671	0.670	0.671	0.001	0.105
6829	1.008	1.049	1.029	0.029	2.819
4710	1.092	1.050	1.071	0.030	2.773
5544	0.765	0.766	0.766	0.001	0.092
7574	0.907	0.927	0.917	0.014	1.542
4712	0.077	0.057	0.067	0.014	21.108
4576	0.408	0.354	0.381	0.038	10.022
7646	-0.574	-0.560	-0.567	0.010	-1.746
T3705	0.925	0.966	0.946	0.029	3.066
#2	1.051	1.023	1.037	0.020	1.909
#5	1.200	1.175	1.188	0.018	1.489
#6	0.161	0.228	0.195	0.047	24.358
#7	0.795	0.785	0.790	0.007	0.895
#8	0.585	0.549	0.567	0.025	4.490
#11	0.702	0.680	0.691	0.016	2.251
#12	0.857	0.859	0.858	0.001	0.165
416	0.699	0.721	0.710	0.016	2.191

Coefficient of intra-assay variability = 5.732

Table 3. Inter-assay values of in-house amyloid beta 42 ELISA Kit

Sample 1				Sample 2			
OD 1	OD 2	OD 3	Mean	OD 1	OD 2	OD 3	Mean
0.706	0.843	0.705	0.751	0.509	0.475	0.478	0.487
0.701	0.713	0.727	0.714	0.534	0.532	0.542	0.536
0.655	0.566	0.658	0.626	0.566	0.567	0.579	0.571
0.652	0.661	0.623	0.645	0.643	0.58	0.598	0.607
0.719	0.701	0.665	0.695	0.651	0.569	0.611	0.610
0.561	1.123	0.513	0.732	0.552	0.489	0.51	0.517

Mean of means of sample 1 was 0.694, sample 2 was 0.555. SD of sample 1 was 0.049, sample 2 was 0.050. %CV of means of sample 1 was 7.086, sample 2 was 8.975

According to Thomsson *et al*. (2014), the value of the coefficient of intra-assay variability is acceptable if the value is less than 10% and the lower the value, the less likely the results are biased due to inconsistencies in the test equipment. Intra-assay value of Aβ 42 in-house ELISA kit can be seen in Table 2.

The coefficient of intra-assay variability of the in-house ELISA kit based on an Aβ 42 monoclonal antibody was less than 10% (Table 2). The test was carried out in one day and used the same ELISA plate. The intra-assay value obtained was sufficient to indicate the stability of the kit and the accuracy of the work performed. Intra-assay precision, known as repeatability, describes the results of repeat tests performed by the same operator and the same laboratory facility, obtaining similar values (Hanneman *et al*.2011). Thus, if an in-house ELISA kit based on Aβ 42 monoclonal antibody is used on different days, with the same operator and place, it is expected to obtain similar or nearly the same values.

The coefficient of inter-assay variability is the accuracy of multiple measurements of samples, controls, and reagents that are analyzed in multiple assays in the same laboratory (Ederveen 2010). Inter-assay accuracy can be determined by repeated measurements under slightly different environmental conditions on the same sample on different days by different laboratory personnel (Ramljak *et al*. 2013). The coefficient of inter-assay variability is a consideration of random errors caused by factors such as laboratory personnel, instruments, and daily conditions during the test.

As shown in Table 3, the coefficient of inter-assay variability of the in-house ELISA kit test based on Aβ 42 monoclonal antibody was 8.032% or <15%. Tests were carried out on 6 plates, on different days, with the same operator. The value of the coefficient of inter-assay variability obtained is sufficient to state the stability of the kit and the accuracy of the work performed. Inter-assay precision, known as intermediate precision, describes nearly identical ELISA results when testing samples using in-house monoclonal antibody-based kits is performed in the same laboratory, by different operators or equipment. Thus, the use of an in-house ELISA kit based on Aβ 42 monoclonal antibody when performed at different times will be quite stable.

CONCLUSION

Detection-based screening using the Enzyme-Linked Immunoassay (ELISA) technique is able to work effectively and accurately in optimizing specific antibody materials or monoclonal antibodies against Aβ 42 peptides. Based on the results and interpretation, inter and intra-assay coefficients, the in-house developed ELISA kit has the potential to detect Aβ 42 from serum.

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