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Comparison of a commercial and a manual antigen coated ELISA tests used in detecting antibodies against Japanese encephalitis virus in pig serums collected from the Province of Bali

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Abstract
The most common serological test perform for screening the antibodies against JEV is enzyme-linked immunosorbent assay (ELISA) and it has been commercially available recently. However, the commercial test tends to be expensive to be used for screening masses samples in developing countries. Then, a manual antigen coated ELISA test was prepared to meet the situation. This study aim was to compared a commercial and a manual coated ELISAs in detecting the antibodies against JEV in pig serum samples collected from Bali, Indonesia. A commercial ELISA was used as a reference to assess its relationship with the manual coated ELISA. A two-by-two table of the two tests' results was set followed by the calculation of a Kappa agreement to determine the relationship of the tests. The results showed a substantial agreement with value of 0.65. However, this substantial value indicated both the tests was satisfactory indicating the manual coated ELISA had similar results compared with the commercial test. Therefore, the precision and validity of tests, especially for the manual coated ELISA need to be evaluated further to ensure it's a good diagnostic essay. In more specific, it can be used to regularly detect antibodies in large amount of the samples in endemic areas of developing countries.

Keywords: Antibodies, comparison, ELISA, diagnostics, Japanese encephalitis

1. Introduction
Japanese encephalitis is a zoonotic born disease, cause by Japanese encephalitis virus (JEV) a member of Flaviviridae, in a group of West Nile virus and Dengue virus [1]. The disease has spread globally, where the higher cases were occurred in Asian countries with around 68,000 cases in human annually happened [2]. In those cases, 20 to 30% with clinical symptom, and 30 to 50 % of the human infection are fatal which can cause long term neurological sequalae [3].
In livestock, the disease can cause encephalitis and death in horses, while in pigs, especially in infected pregnant sows, they may experience reproductive disorders, such as abortion, mummification, or stillbirth [4, 5]. Pigs are reported to be the amplifying hosts of the JEV, where the virus can be amplified in the infected pigs' body before it shades through oronasal exudate. Alternatively, the virus can be brought by the mosquito vector that feed on them from the viremia phase of the infected pigs [6]. Surveillance diagnostic of infected pigs can be used to indicate the risk of the JEV circulation in the environment as well as the risk of the disease transmission into public health around.
Many options can be used in detecting the disease which can be conducted with molecular or serological assays. In the diagnostics test for JEV infection confirmation, it can be performed by using the genetic sequence through reverse transcriptase polymerase chain reaction (RT-PCR) to identify the viral genetics [7]. Nevertheless, developing countries tend to have a problem with a limited access to detect some diseases based on the diagnostic tools in their regions. In the JEV detection, it requires high diagnostic technology to screen the disease in the areas. Molecular diagnostics like polymerase chain reaction (PCR) requires not only highly skilled human resources, but also high technology infrastructure and expensive stuff to run the test [8].

However, ELISA is mainly used in detecting the antibodies that regularly used for screening test performed to detect the antibodies of JEV in pigs [9] even though it has a likelihood of cross-reaction with other Flaviviruses' antibodies [10]. Even so, the ELISA test is one of the diagnostic test can be performed for surveillance of the antibodies detection and its recommended by the world health animal organization OIE [11]. Recently, the commercial ELISA tests are available. However, the price tends to be high and it could be a problem for conducting the diagnostics for many sample population run in developing countries.

The role of serological tests, like ELISA are also important part of diagnostic tools that can be used to screen the infection. Even though standardized ELISA kits have been commercially available, they were limited and expensive. Therefore, a manual self-coated ELISA has been developed to be used to screen the antibodies against JEV in Indonesia, including Bali where the cases of JE in humans reported to be nationally high. This study aim is to compare the two diagnostic tests between a standardized porcine ELISA commercial kit and a manual self-coated ELISA in detecting the antibodies against JEV.

2. Material and Methods

2.1 Samples collection

Ninety two serum samples were collected randomly from the smallholder pig farmers from three different cluster areas in the province of Bali. List of the farmers was required from the local authorized animal health. One to two mL of the blood was collected from the jugular vein of the sampled pigs. The collected blood was clotted until the serum released. The serums were placed in a 1.5 mL tubes before transported with dry ice in an icebox to the lab and stored them in a – 20^{0}C freezer. One day before the serum being tested, they were stored in a refrigerator.

2.2 A commercial ELISA test

A commercial porcine antibody JEV ELISA kit was used as a representative of the standardized test in comparison with the manual antigen coated of ELISA. The same number of 92 pig serum samples were used in both tests. The commercial ELISA kit test used reported to have sensitivity (Se) of 98.3% and specificity (Sp) of 98.2% (E-AD-E002 Porcine Japanese encephalitis virus antibodies ELISA kit, Elabscience, China). Thirty minutes before using, the kits and the serums were stored in room temperature (approximately 25-27^{0}C). A microplate kit comprised with 96-well of microtiter plate pre-coated with recombinant JEV E2 antigen, horse radish peroxidase (HRP) conjugate, positive and negative controls, sample diluent, stop solution, and the substrate reagents. Two wells used for controls (each for positive and negative controls), another two wells was blank, and the other wells were used for the serum samples tested. Five microliters of each serum sample were added to the microplate wells. Then, a 195 microliter of the sample diluent was added to the sample wells, positive and negative controls, and the blank wells. The microplate then was covered by a plate sealer and incubated the microplate at 37^{0}C for 30 min in shading light. Next, the liquid in each well was removed, 300 µL of washing buffer was poured to each well in the microplate and wash them for five times. In the last wash, the plate was inverted and patted them against thick clean absorbent paper. After that, a 100 microliter of horse radish peroxidase (HRP) conjugate was added into each wells, except into the blank well. Then the plate was covered and again incubated it at 37 ^{0}C for 30 min in shading light. After the incubation, the plate was washed with the same washing procedure before. One hundred microliter of subtract reagent was then added into each well, before the plate was then covered by the plate sealer and re-incubated it again at 37 ^{0}C for 10 min in shading light. After the incubation, the wells in the plates were added with a 50 µL of stop solution. Finally, the optical density (OD) values were read with 450 nm wavelength of an ELISA reader machine (ELx 800-Biokit).

Based on the ELISA kit manual, the serum samples assigned to be positive containing antibodies against JEV when they had OD values equal or greater than 0.380. However, for the samples that had OD value less than 0.2 was categorized negative containing the antibodies and the OD value between equal to and greater than 0.2 and less than 0.38 was suspicious. The positive and negative results of the samples were included to be analyzed in the study, whereas the suspicious samples were excluded.

2.3 A manual antigen coated ELISA test

Meanwhile, the manual self-coated ELISA was prepared based on the ELISA preparation adapted from Adi *et al*. (2016) [12]. A concentration of 0.5 ng/mL of the JEV antigen was made by mixing 7.5 µL JEV (recombinant protein, Creative Diagnostics product) and 15 mL carbonate bicarbonate coating buffer (0.1M carbonate buffer, pH 9.6). Coating was performed by filling 75 µL of the antigen dilution into each well of the 96 well microplates. The plates were sealed and incubated for 16 h at a temperature of 4^{0}C. Then, the JEV coated microplates were washed three times by using Tween washing solution 0.01%. Blocking was conducted by adding 200 µL of 3% non-fat skim milk (Oxoid product) blocking buffer each well. Then, cover the plates with adhesive plastic and incubated them for an hour in incubator at 37^{0}C. Next, the plates were washed three times by using the washing buffer. A 50 µL of 3% skim milk and 50 µL primary antibody in each well were added. Next, the plates were again covered with adhesive plastic and incubated them for 1 h in incubator at 37^{0}C. The plates were then washed three times with the washing buffer. Conjugate secondary antibody was added at 100 µL. Then the plates were again covered with adhesive plastics and incubated them for 1 h at 37^{0}C. The plates then washed three times with the washing solution. A 50 µL substrate solution (TMB peroxidase substrate A and Peroxidase substrate B were mixed) was put into each well. Finally, after 15-20 min, a 50 µL stop solution (phosphoric acid) was added to the wells before the absorbance at 450 nm was recorded on the ELISA plate reader (ELx 800-Biokit). Cut off titre value of the ELISA test was determined by calculating the mean of all negative controls plus 6 times standard deviation (SD) of the negative controls. The serum was assigned to be positive containing the antibodies against JEV, when it has the OD value greater than the cut off, while the serum sample that had the same and lower than the mean value of the negative controls was assigned to be negative.

2.4 Animal ethics

This protocol research has been approved by the ethics commission for the use of animals in research and education, Faculty of Veterinary Medicine, Udayana University with certificate reference number 14/UN14.12.9/PD/2020.

2.5 Kappa agreement reference

Performance of the manual coated ELISA was compared with the commercial ELISA kit by calculating the Kappa agreement score to evaluate the proportional agreement of the test. The values of the Kappa score were adapted from Landis & Koch (1977) [13], in which the value of Kappa score is low when the agreement value is under and equal to 0.22. Meanwhile, the agreement is fair, when it has the Kappa score between 0.21 to 0.40; moderate for the score between 0.41 to 0.60, substantial for the score between 0.61 to 0.80, and almost perfect for the score between 0.81 – 1.0).

3. Results and Discussion

3.1 Results

A total of 92 pig serum samples were used in this study analysis. The serums were collected from the three areas in Bali, such as 31 samples collected from Badung regency, 29 pig serums from Denpasar city, and 32 pig serums from Karangasem regency (table 1).

Table 1: Quantity and the three cluster areas of the pig sample collection.

Number and areas of the pig serum sample collection			
Number	**Cluster I**	**Cluster II**	**Cluster III**
1	B1	D1	K1
2	B2	D2	K2
3	B3	D3	K3
4	B4	D4	K4
5	B5	D5	K5
6	B6	D6	K6
7	B7	D7	K7
8	B8	D8	K8
9	B9	D9	K9
10	B10	D10	K10
11	B11	D11	K11
12	B12	D12	K12
13	B13	D13	K13
14	B14	D14	K14
15	B15	D15	K15
16	B16	D17	K16
17	B17	D18	K17
18	B18	D19	K18
19	B19	D20	K19
20	B20	D21	K20
21	B21	D22	K22
22	B22	D23	K23
23	B23	D24	K24
24	B24	D25	K25
25	B25	D26	K26
26	B26	D27	K27
27	B27	D28	K28
28	B28	D29	K29
29	B29	D30	K30
30	B30	0	K31
31	B31	0	K32
32	0	0	K33

When the tests performed and the OD values measured, the range of ELISA OD values resulted from the commercial ELISA kit were higher compared to the manual antigen coated ELISA test. Although the minimum OD values of both tests were found almost the same at 0.1 which indicating no likely containing antibodies against the JEV, the highest reached more than 3.5, while the highest OD value of the manually coated ELISA was observed under 2.0 (Figure 1).

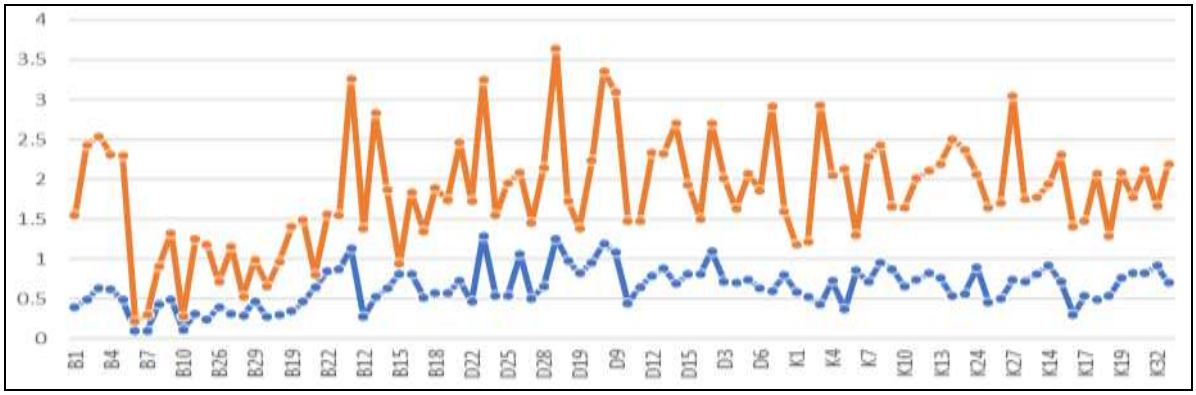

Fig 1: Chart of the OD value results in comparison between the manual antigens coated ELISA and the commercial ELISA kit.

*The orange lines and points are the OD value results in the samples tested using the ELISA Kit, whereas the blue lines and points are the OD value results in the samples tested using the manual antigen coated ELISA.

Based on the results of the two ELISA tests, they were put into a two-by two table (Table. 2) for the calculation of the Kappa agreement that illustrated in the two-by-two table (Table. 3) with the formula provided.

Table 2: A two-by-two table of the two tests results from the same serum samples tested by using a commercial ELISA test as a reference in comparison with a manual antigen coated ELISA.

		A commercial ELISA		
		Positive	**Negative**	**Total**
Manual antigen coated ELISA Test	Positive	78	1	79
	Negative	6	7	13
	Total	84	8	92

Table 3: The relationship of the two ELISA tests results into a two-by-two table before calculating the Kappa coefficient agreement.

		A commercial ELISA		
		Positive	**Negative**	**Total**
Manual antigen coated ELISA	Positive	a	b	a + b
	Negative	c	d	c + d
	Total	a + c	b + d	(n) = a + b + c + d

The Kappa coefficient agreement (*K*) was calculated using formula [14]:

$$K = \frac{[(a+d)/n] - \{[(a+b)\times(a+c)/n + (c+d)\times(b+d)/n]/n\}}{(1 - \{[(a+b)\times(a+c)/n] + [(c+d)\times(b+d)/n]/n)\}}$$

Kappa coefficient value = 0.65, which means a substantial agreement observed between the two tests.

3.2 Discussion

Japanese encephalitis is an endemic disease in Indonesia, and the related cases in humans is nationally the highest number among other provinces in the country. Similarly, the cases in animals is also suspected to be high as the dominated amplifying hosts were acted by pigs and the virus is spread by *Culex* spp. mosquitoes as the vector in Bali [15, 16].

However, the diagnostic of the disease is screened mainly using serological tests for detecting the antibodies against the JEV in both humans and animals. There are many references of serological diagnostic tests that have been widely used in detecting the antibodies against JEV, such as: hemagglutination inhibition (HI), indirect fluorescent antibodies test (IFAT), latex agglutination test (LAT), and also ELISA [17]. Although more sensitive and specific of molecular diagnostic tests have already available recently, the ELISA test is more commonly used for the diagnostics and it is still recommended by the World Health Organization (WHO) for human cases [18] and OIE for the animal cases [11]. The ELISA test can be used to detect the certain antigen or its antibodies including the JEV.

Similarly, ranges of antigen and antibodies types are also available in the ELISA test depending on aims of the diagnostics. However, in this study the immuno-globulin G (IgG) antibody against JEV was detected in both tests in relation with the cross-sectional study performed to assess the serological proportion of the IgG in pigs in the study area. Even though the IgM antibody detection is also available and may be used to detect several early infection, the IgG can be detected after around a week or more of the infection. The IgG also persists longer in the body. It is beneficial to be used to detect the infection in animals where the vaccination against JEV has not yet implemented. As no vaccination program against JEV in animal in the study area, detection the IgG may give an indication about the rate of the pig infection or even risk of the disease that may transmit to humans in the study area.

At this moment, the porcine Japanese encephalitis virus ELISA kit test has already and commercially been available. However, in the JE endemic developing countries, providing the commercial kit may require excessive fund and in certain situation, it could also be difficult to obtain in the countries. In consequence, a manual coated ELISA test needs to be developed to meet the diagnostic standard and pass the limitation of the research funding availability.

In this study, standardized of the commercial ELISA kit was assigned to meet the standardized diagnostic test that compared with a manual antigen coated ELISA test to assess whether the manual antigen coated ELISA test has a proportional agreement with the commercial ELISA kit. The results of the manual antigen coated ELISA test that developed and used indicate no difference compared to the results of the standardized commercial ELISA test used in this study. This may suggest that the manual coated of the ELISA test can be used for the screening test in detecting the antibodies against JEV.

The benefit of using the manual coated ELISA not only produced in the similar results with the standardized of the commercial test, but it is also more economical as they could save more money. This simple and efficient diagnostic tool is more likely feasible to be implemented in the JE endemic developing countries to support the disease survey as it is not only has relatively high sensitivity and specificity, but it is also reproducible and affordable [19].

In general, in term of using ELISA for detecting antibodies against JEV, the ELISA test is still being used worldwide, especially in the countries where the molecular diagnostics have not been available. Instead of a simple and efficient diagnostic tool, ELISA is an alternative to be used for screening the large number samples rapidly [9] and beneficially use in global epidemiological surveys with large amount of samples [20].

Out of the beneficial in using the manual antigen coated ELISA, the drawbacks of the manual ELISA require more time in the application of the test. For example, for coating the antigen in the ELISA microplate, an over-night or around 16 hours are needed for the incubation [12]. In addition, some

solution of the washing buffer, positive and negative controls, and/or sample diluent also need to be provided which require more time to spend. However, in the commercial kit, those stuff have already provided so that it can save much more time.

Moreover, the manual coated ELISA also requires more advanced lab skill to prepare the test. In the manual antigen coated ELISA, specific dilution of the antigen have to be prepared before it is going to be coated in each of the microplate well. Coating the diluted antigen in each well of the ELISA microplate needs specific skill in performing it as well. Meanwhile, in the commercial kit ELISA, the microplate has already been coated. Then, only the samples mainly need to be prepared for conducting the test. This is the main reason of the commercial is much more saving time in performing the test.

Based on the analysis of kappa agreement test performed the manual coated ELISA test had a good proportion agreement with the commercial ELISA test. This suggesting that the manual ELISA test was in agreement with the standardized commercial ELISA kit. Even so, both of these test may indicate good diagnostic tests or conversely, they may also be the inferior tests. Similar results was observed in comparing the ELISA tests with another serological test, virus neutralization test (VNT) which had the kappa 0.6 indicating both tests had a good agreement [10].

Even though using ELISA in detecting the JEV antibodies may be cross reacted with other flaviviruses' antibodies, a more validated diagnostic tests like virus neutralization (VN) test or plaque reduction neutralization test (PRNT) can be performed to confirm the results. However, these two tests performance requires live viruses and therefore, the minimal laboratory of Biosafety level III is also needed [21]. This lab is limited available in developing countries. As a results, the diagnostic tests may be difficult to be performed. In fact, in relation to the JEV antibodies detection, both of the tests between ELISA and virus neutralization have a good correlation coefficient at 0.8 with sensitivity and specificity of 82% and 98% respectively which suggesting that they have a good similar results in detecting the antibodies [22]. This also indicate the ELISA test is also a qualified diagnostic test.

A substantial agreement observed between the two tests in this study indicating the substantial relationship between the tests even though the relationship did not necessarily mean that both of the tests were good. It can be a way around, the two tests could also mean inadequate for the diagnostics. However, more evaluation of the diagnostic essays is required in order to provide good diagnostic tests. Therefore, more analysis for the tests' precision and validity are needed to be more confirmed of tests being used whether they are good and validated in using the screening test for the diagnostics [23]. Next study on the precision and validity of the tests may support the quality of the tests being used for the screening which may result in the reference tests to be used to detect the disease in the areas of developing countries.

4. Conclusion

Both of the commercial ELISA test and the manual coated ELISA test have a good agreement, which indicate they have the similar results in detecting antibodies against JEV from the pig serum samples collected in Bali. However, in some cases, they also have some difference that need to be considered when using them for the diagnostics test, especially in the tests' preparation. Both of the tests may be used for the detection of antibodies against JEV, especially in endemic areas for the mass sample detection.

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