

COMPARING ANTIBODY TITERS AFTER VACCINATION IN DOGS USING ELISA READER WITH IMAGE PROCESSING TECHNIQUES

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Abstract

Rabies is a zoonotic diseases that is caused by a virus of the genus Lyssa virus that attacks the central nervous system (CNS) and is transmitted through the bite of Rabies Transmitting Animals, especially dogs. Rabies can be prevented but is always fatal in humans if it infects the Central Nervous System (CNS). Vaccination has been used as one of the rabies prevention programs. A total of 83 samples were tested using Indirect ELISA (Enzyme-Linked Immunosorbent Assay) to identify antibody titers after rabies vaccination. Antibody titer correlated with absorbance value and concentration of standard solution. The absorbance value was read by ELISA reader and desktop scanner. The absorbance values were read at 450 nm and 620 nm as a reference using an ELISA reader and images from a desktop scanner were processed using the ImageJ application. The purpose of this study was to compare the obtained results using an ELISA reader as the gold standard and scanner images processed using ImageJ including sensitivity, specificity, and accuracy. There is no significant difference between the ELISA reader as the gold standard and alternative test equipment. The desktop scanner has a sensitivity of 97%, a specificity of 88.8% and an accuracy of 96%. The absorbance measurement method using an ELISA reader is expensive and difficult relatively to carry out in a laboratory with minimum financial support so that the image from desktop scanner processed using the ImageJ application is expected to be an alternative choice for an ELISA (Enzyme-Linked Immunosorbent Assay).

Keyword: rabies, vaccination, antibody titer; ELISA reader; desktop scanner

INTRODUCTION

Rabies is a zoonotic disease Rabies has been known for centuries, its spread is almost all over the world. According to WHO data, rabies occurs in 92 countries and is endemic in 72 countries. Rabies is an important acute encephalomyelitis disease that is greatly feared by the public because it is fatal in all warm-blooded animals (dogs, cats, monkeys, horses, buffalo, cattle, sheep, goats etc.) included human. Rabies is caused by a neurotrophic virus of the genus Lyssavirus Family Rhabdoviridae which has evolved to target neurons as part of their normal infection cycle. Almost all human infections are caused by the bite of Rabies Transmitting Animals, especially dogs (Rahayu 2010). Bat bites are also a source of



infection but are responsible for a small number of cases. This means that technically simple rabies control is controlling rabies in dogs. The rabies virus can spread from the bite site of a rabid dog, through the terminal endings of motor neurons that innervate muscles and travel along axon fibers to nerve cell bodies, throughout the central nervous system and to the salivary glands where the rabies virus can be easily transmitted to the host. other.

Based on the Ministry of Agriculture in Kepmentan No.4026/Kpts/OT.140/04/2013, rabies in Indonesia is a strategic infectious animal disease. According to Directorate of Animal Health (2007), one of the efforts to control rabies that can be done is mass vaccination. Vaccination coverage of at least 70% of the dog population should be acquire immunity to eliminate or prevent rabies outbreaks (WHO 2005).

The success of vaccination in animals or humans can be tested using Enzyme-Linked Immunosorbent Assay (ELISA). The ELISA method is one of the methods used to detect rabies antibodies in animal serum (dogs) as well as in human serum (Lequin 2005). The ELISA method is also very useful for detecting antibodies associated with epidemiological surveys in large population sizes (Xu et al., 2007). This antibody titer is used to confirm the antibody response after vaccination in dogs (Setiaji and Agustini, 2011). Antibodies are special chemicals capable of binding to specific antigens. Specific antibodies can be measured using predefined antigens and this is the basis in various diagnostic biological tests including ELISA. The results of the ELISA test, obtained from absorbance measurements using an ELISA reader. The working principle of the ELISA reader is based on colorimetry, namely the intensity of light absorbed in a colored solution with a certain wave is the absorbance value that is read. The ELISA reader is an expensive tool and is difficult to provide in laboratories or universities that have minimum funds. In addition, the maintenance of this tool is relatively difficult and less practical for reading ELISA results in remote places. In this regard, an image processing method was developed using ImageJ software based on colorimetry by taking sample images recorded using a desktop



scanner. This method is considered cheaper, easier, faster, and more practical, and is expected to be an alternative choice for ELISA readers commonly used today. The purpose of this study was to compare the ELISA reader as the gold standard and a desktop scanner which includes sensitivity, specificity, and accuracy of the antibody titer after rabies vaccination.

METHOD

This study used blood samples collected from the cephalic antebrachii vein of dogs in Cisolok and Jampang Tengah sub-districts in Sukabumi district. Blood samples were taken after vaccination. Blood is coagulated to produce serum and then stored in a mini tube. Serum was transported through a cold chain (temperature 4-8 °C) using a cool box containing ice blocks until measuring process of antibody titer in integrated laboratory of Faculty of Veterinary Medicine of IPB University. Prior to testing the serum antibody levels, the samples were inactivated using a water bath for 30 minutes at a temperature of 56 °C.

The test procedure was carried out according to the work manual for the Indirect ELISA Rabies Demeditec® kit (Demitec Diagnostics GmbH). The first thing to note is making a recording sheet. The purpose of making a recording sheet is to determine the location of the control and serum samples in the microplate well.

A total of 1 ml of negative control (grey cap) was dissolved in 1 ml of aquabidest and 0.5 ml of positive control (purple cap) was dissolved in 0.5 ml of aquabidest. Dilutions were performed on the positive control and serum samples. Serum samples were diluted with ELISA buffer as much as 1:100. Positive control dilutions using ELISA buffer were carried out in a ratio of 1:50, 1:150, 1:450, 1:1350 until the concentrations obtained from positive control dilutions were 1.6, 0.8, 0.4, and 0.2 EU (Equivalent Units). 100 µL of this solution was transferred, using a micropipette, to a microplate that had been coated with antigen



according to the recording sheet. This was done for all solutions, namely positive control and samples, leaving two wells for substrate control.

100 µL ELISA buffer was introduced into wells A1 and A2 as a control substrate. The microplate was then covered with absorbent plastic and incubated at 37 °C for 60 minutes. The absorbent plastic was opened and the liquid on the microplate was removed, then washed 3-5 times using a washing solution with a volume of 125 µL into each well. The washing solution was PBS Tween 20 with a concentration of 0.5%. The microplate is tapped on a tissue that can absorb liquid so that there are no bubbles in the well. The antibody-antispecies conjugate in this test using HRP (Horse Radice Peroxidase) was added to each well as much as 100 L. The microplate was covered with adsorbent plastic and incubated at 37oC for 60 minutes. The liquid is removed and re-washing is carried out according to the previous washing procedure. The mixing of substrate A (white cap) and substrate B (blue cap) was carried out right before use and homogenized. Furthermore, the addition of 100 l of substrate solution into each microplate well was carried out for 20 minutes in a dark room at a temperature of 21 C dark. A total of 50 l of stop solution was added to each well and the solution was homogenized, then observed for color changes and immediately read with an ELISA reader.

The ELISA results were read using an ELISA reader with two filters, namely the wavelength of 450 nm and 620 nm as a reference. The results of readings using an ELISA reader are in the form of OD (Optical Density) or absorbance values. Absorbance is presented in the form of a standard/calibration curve to determine the relationship between concentration and absorbance. Linear equations from standard/calibrated curves made using Microsoft Excel will result in the equation $y = a+bx$. The y-axis is the absorbance value and the x-axis is the concentration of the standard solution that has been obtained, namely 1.6, 0.8, 0.4, and 0.2 EU (Equivalent Unit). The values of a and b are constants obtained from the calculation of the intercept and slope and can be used as a reference in calculating the antibody titer of the sample with the formula $x= y-a/b$. The

final result of the ELISA test is expressed in EU equivalence (Equivalent Units). The interpretation of the results of the EU sample 0.5 EU indicates a positive result or a protective rabies antibody titer, while the EU sample < 0.5 EU indicates a negative result or the rabies antibody titer is not protective (Yang 2014).

After using ELISA Reader colored samples in the microplate were taken with a desktop scanner equipped with a light source from Scanjet XPA which was placed on the microplate. The scanned image is then saved in the tagged image file format (TIFF) on the laptop. (Figure 1).

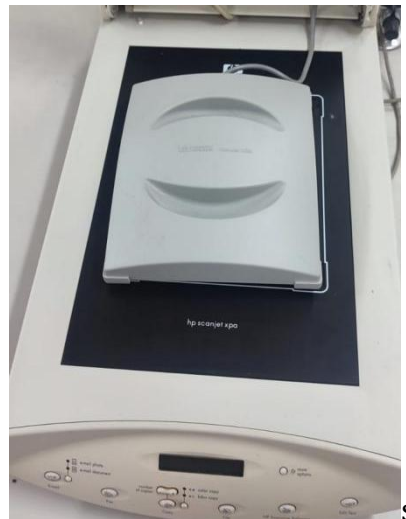


Figure 1 HP Desktop scanner equipped with Scanjet XPA

The images that have been saved are then processed using image processing software imageJ. ImageJ represents colors in pixels and can be converted to numbers in red-green-blue (RGB) color components. Color intensity can be translated into absorbance by Lambert-Beer law, namely:

$$A = -\log \left(\frac{I}{I_0} \right)$$

A = absorbance value,

I = intensity of each red, green, and blue channel



I_o = is the maximum value of a pixel, which is 255 (Soldat et al. 2009).

Furthermore, the absorbance and concentration values are presented in the form of a standard/calibration curve so that the linear equation $y=a+bx$ is obtained. Values a and b are used as constants in the calculation of antibody titers in samples.

Data analysis was performed using a diagnostic test with test parameters of sensitivity, specificity, and accuracy. According to Mandrekar (2010), the formula for sensitivity = $a/(a+c)$, specificity = $d/(b+d)$, and accuracy = $a+d/(a+b+c+d)$, where a is a true positive / true positive, b is a false negative / false negative, c is a false positive / false positive, and d is a true negative / true negative.

RESULTS & DISCUSSION

Measurement of Standard solution reading using ELISA reader

Antibody titers after rabies vaccination in dogs in Sukabumi, West Java were obtained from the calculation of the concentration and absorbance of standard solutions (positive control). The absorbance value is the value obtained from reading the standard solution using an ELISA reader, while the concentration value is the value obtained from the diluted standard solution. The standard curve obtained from the readings of the ELISA reader can be seen in Figure 1.

Figure 1 shows a linear regression graphic that represents a linear relationship between absorbance and standard solution concentration from the readings of the ELISA reader. The linear regression equation is $y = bx+a$ which is shown in Figure 1, namely $y = 0.5781x - 0.1025$. The y-axis is the result of the reading of the ELISA reader in the form of absorbance values, while the x-axis is the value of the concentration of the specified standard solution.

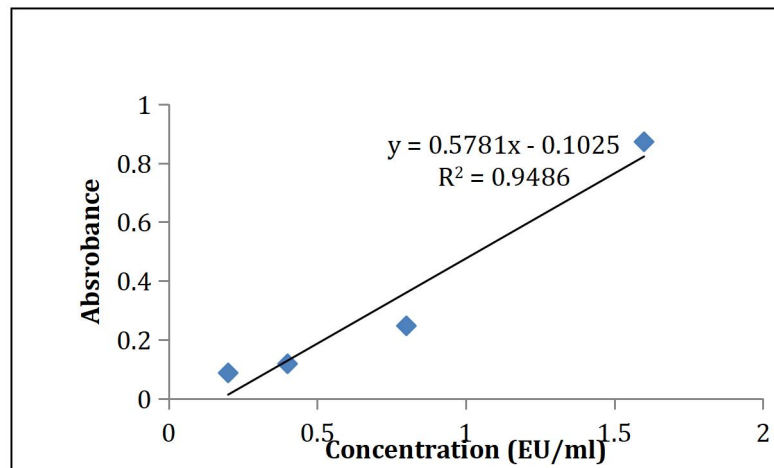


Figure 1. Relationship between absorbance and concentration of standard solutions using ELISA reader

The value of a is the intercept or the intersection with the vertical axis and b is the slope or gradient. According to Bluman (2012), the formation of a linear line on the graph even though it is not perfect, shows that the concentration and absorbance are linearly related.

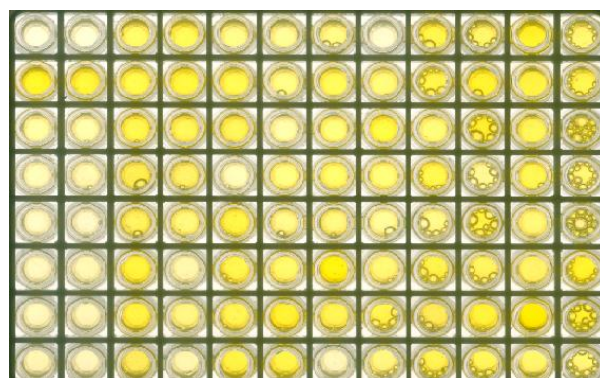
The value of R^2 in Figure 1 is used to calculate the effect of the concentration value on the absorbance value. According to Walpole (1993), simple linear regression analysis aims to determine the effect of the independent variable on the dependent variable, in this case the independent variable is the concentration value and the dependent variable is the absorbance value. The value of R^2 is in the range of 0 to +1. The R^2 value in Figure 1 is 0.9486, which means that 95% of the factors that affect the absorbance value are the predetermined standard solution concentrations. According to Skoog et al. (2007) based on Lambert-Beer Law, the higher the concentration value, the higher the absorbance value.

The strength of the relationship between concentration and absorbance can be seen from the value of the linear correlation coefficient denoted by R. The value of R is obtained from the root of R^2 . According to Bluman (2012), the value of R will have a strong relationship if it is close to the value of -1 for a negative correlation or +1 for a positive

correlation. Figure 1 shows the value of R^2 is 0.9486 which means the value of R is 0.9370. R value close to +1 indicates a strong positive relationship between concentration and absorbance. The standard curve of the reading using an ELISA reader can be used as a reference in determining the antibody titer of the sample being tested.

Image processing from desktop scanners using the ImageJ.

The amount of light absorbed by a colored solution is called absorbance. Absorbance measurements can be known through image processing with imageJ which is scanned using alternative tools, namely desktop scanners. Data acquisition using a desktop scanner produces an image as shown in Figure 2.



Standard Sampel

Figure 2. Image of a microplate filled with standard and sample solutions using a scanner.

Acquisition data using a scanner and cellphone camera that produce image of micoplate filing with standard and sample solution was analyzed colorimetrically using application, namely ImageJ. The color of the solution in Figure 2 display bright to thick yellow. According to Underwood and Day (2002), if the concentration of a colored compound in a solution is higher, the solution will be more concentrated in this color and

will have higher the absorbance value. The visible color is a collection of several basic colors. According to de Moraes and de Lima (2014), image analysis of the colorimetric reaction on the microplate produces red, green, and blue basic colors, each of which is grouped into each microwell. The average of the clustered colors will be transformed using Lambert-Beer's law into absorbance values.

According to Soldat et al. (2009), the central mean value for clustered colors is the result of selection in the bottom area of the well which has relatively the same color and is taken using the ovale selection function in the ImageJ application with the same diameter. The absorbance value obtained is used to create a standard calibration curve using one of the colors between red, green or blue (de Moraes and de Lima 2014). The following is a calibration curve for a standard solution for image processing using a scanner and mobile camera, which can be seen in Figure 3.

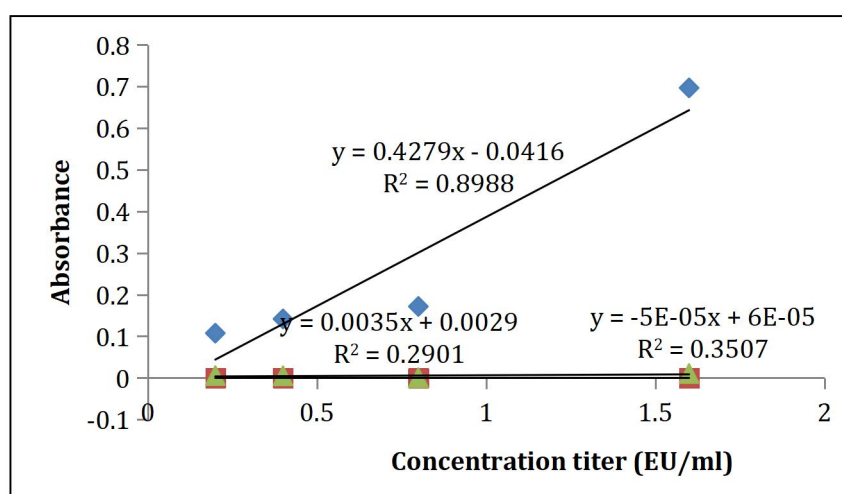


Figure 2. Relationship between absorbance and concentration of standard solutions using Desktop Scanner

The blue channel shows a linear slope and the highest R² value is 0.8988 when compared to the green and red channel which are only 0.2901 and 0.3507 (Figure 3). According to Walpole (1993), if the value of R² is increasing, the concentration value will have a greater effect on the absorbance value.. According to Soldat et al. (2009), an

increasing slope of a linear curve will cause a change in the value on the x-axis (concentration) will increasingly affect the change in the value on the y-axis (absorbance. According to Rusmawan et al. (2011), the absorbance value of the red, green, or blue channel components which have very small values (close to zero) cannot be used to measure a concentration in the tested sample, in this case the green and red colors cannot be used to measure antibody titers. sample.

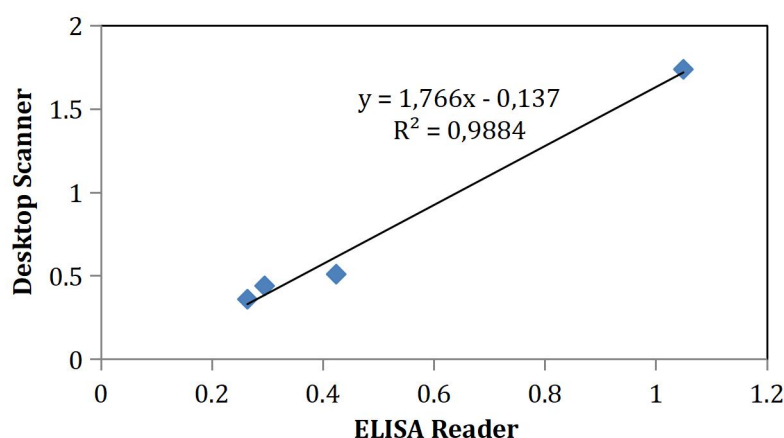


Figure 4. Standard solution correlation between scanner and ELISA reader based on standard solution measurement results

Correlation curve is made to measure the relationship between scanner with ELISA reader. The following is the correlation curve of the standard solution of scanner with ELISA reader as gold standard (Figure 4).

Figure 4 shows a linear relationship between the standard solution which was read using a desktop scanner to the ELISA reader. The strong relationship between the test equipment can be seen from the values of R^2 and R to the gold standard. Figure 4 shows that the scanner have a strong relationship, as seen from the R^2 value between the scanner to the ELISA reader, respectively, which is 0.9884. This value is very high and close to +1. According to Bluman (2012), a high R^2 value (close to +1) indicates the strength of the relationship between variables on the x and y axes.



Measured antibody titer after vaccination is categorized into immunity level, namely protective titer and non-protective titer against rabies. The results of the calculation of antibody titers from desktop scanners were compared with an ELISA reader using a diagnostic test. According to Tumbelaka (2002), the diagnostic test is used to compare the results of the allegations/predictions of an examination or test against a standard value that is close to the truth/gold standard. The following are the results of the scanner diagnostic test compared to the ELISA reader as the gold standard (Table 1).

Tabel 1. Comparative diagnostic test results between scanner and ELISA reader (agold standard)

Hasil uji alat	Positif E	Negatif E	Jumlah
Positif S	72 (a)	1 (b)	73 (a+b)
Negatif S	2 (c)	8 (d)	10 (c+d)
Total	74 (a+c)	9 (b+d)	83 (a+b+c+d)

Note: S = Desktop Scanner, E = ELISA Reader, a = true positive, b = false negative, c = false negative, d = true negativ

Table 1 shows total number of samples tested using the ELISA reader as the gold standard and 83 samples of image processing from Desktop Scanner. The number of positive samples on the ELISA reader reading was 74 samples and the number of negative samples was 9 samples. The high number of positive samples occurred because the samples used were post-rabies vaccination data so that protective antibody titers had been formed. The result of the sensitivity value of the scanner test tool is 97%. This sensitivity value is high, referring to Thrusfield (2005) that the confidence interval for a very high sensitivity or specificity value is >95%, while the lowest confidence interval is <5% of the total population.

Tumbelaka (2002) argues that a high sensitivity value is influenced by a high true positive value and a low false negative value. This indicates that image processing from desktop scanner using ImageJ has a good tool capability in detecting positive antibody titers based from the high true positive value of the scanner reading against the ELISA



reader, although there are still false negative values. The results of the specificity value tend to be high, namely 88.8%, but this value is still less than 95%. This happens because the number of negative samples is too small. Table 1 shows that the true negative antibody titer readings with the scanner test equipment were 8 samples out of 9 samples detected as negative by the ELISA reader. A total of one sample was detected as false positive from the scanner test equipment. According to Maxim et al. (2014), the consequence of a false positive value is the detection of a positive result from the test equipment but the actual value is negative. This needs to be considered in the detection of antibody titers if the test equipment used still has false positive results because the antibody titer read by the scanner is positive while the actual antibody titer is not protective (negative).

High sensitivity and specificity values are followed by high accuracy and validation values. The validity of a tool is based on the accuracy of the test equipment (scanner) in detecting the vaccination response, both positive antibody titers and negative antibody titers to the ELISA reader. According to Betz et al. (2011) accuracy is the closeness of the experimental test results to the actual value. The accuracy value is the value of the total true positive (true positive) and true negative (true negative) test results. The accuracy value of the scanner is quite high, namely 96%. This accuracy means the desktop as test tool is able to read the number of antibody titers either positive or negative with a value close to the reading from the ELISA reader (gold standard).

CONCLUSION

Readings of vaccination responses based on antibody titers with digital image processing from desktop scanner have high sensitivity, specificity, and accuracy values. This shows that image processing using desktop scanner has a reading close to the ELISA reader (gold standard).

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