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RESEARCH ARTICLE

ELISA METHOD TO DETECT ABO BLOOD GROUP IN EXTERNAL SECRETION FLUIDS

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ABSTRACT

Background: Usually it takes a large number of volume sample to determine blood group from external secretion fluids. But, in certain condition, samples are only available in very small amount. The objective of this study is to detect the presence of ABO blood group substances in mucosal fluid using ELISA technique, thus only requires small amount of samples.

Objective: To develop an ELISA technique using the current anti-ABO antibodies for determination of blood group by hemagglutination technique and second peroxidase label antibody specific for mouse IgG, originally used for another ELISA technique.

Methods: 100 µl of diluted human intestinal mucosal fluid were incubated overnight in 4°C in ELISA microplate wells, followed by addition anti-ABO antibodies. Then after incubation, a second revealing antibody anti mouse IgG labeled with peroxidase was added. After a brief incubation, substrate H_2O_2 and chromogenic TMB were added.

Results: Positive reaction is marked by development of blue colour, which, on termination enzymatic reaction by addition 100 µl H_2SO_4 change to yellow.

Conclusion: An ELISA method for detecting ABO substance in mucosal fluid can be developed from antibodies not specifically made for this technique, but specific only for the target.

Keywords : ABO blood group substance, Antibodies, ELISA, Chromogenic substrate

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INTRODUCTION

Individuals can be classified as secretor or non-secretor based on their ability to secrete ABO blood group in the external body fluid.[1] Secretor individuals are the ones that secrete blood type antigens into external body fluids. On the other hands, non-secretor individuals are those that do not secrete or secretes a very small amount of blood type antigens into their external body fluid.[2]

Approximately 60% of the population are secretors that secrete ABO blood group antigens into external body fluids such as saliva, digestive tube juice, nasal and respiratory tract mucous, and also seminal plasma.[3] The importance of detecting blood group in external body fluid is to determine blood group of a person without fingertip puncture (non-invasive technique) in forensic investigation.

The usual technique that is used to determine blood group in external body fluid is by hemagglutination or absorption inhibition method.[2,4] The inconvenience of this technique is that it requires a relatively large number of volume sample. Often, mainly in a forensic investigation, samples are available in small quantity. This condition may limit blood group determination using hemagglutination or absorption inhibition technique, as it is less sensitive using small samples.

The objective of this study is to develop a method to determine ABO blood group by using only a small amount of sample. *Enzyme-linked immunosorbent assay* (ELISA) can be used to detect any compound specifically and it can be performed with small amount of sample without loss of sensitivity. As an immunochemistry technique, it uses specific antibody to detect specific antigens. In this research, antibodies agglutinin anti-A and agglutinin anti-B, usually used for detecting ABO blood group in the blood were used to detect ABO blood group substances in the external body fluid. A second antibody, specific to agglutinin A and B were then added to bind the agglutinin antibodies which had bound the ABO substance specifically. The second antibody was labeled by conjugating it chemically with peroxidase enzyme. The presence of antigen-antibody complex is then revealed by addition a suitable chromogenic substrate to peroxidase. The enzyme-substrate reaction will give a colour which indicated the presence of the immune complex, hence the antigen i.e ABO substances. Since it uses enzyme amplified antibody to detect the sample antigen, this reaction is much more sensitive and can be used with only a small amount of sample.

MATERIAL AND METHODS

Materials

This study used ELISA method to detect blood group antigen. Samples were taken from lumen of human intestines. These were taken from 32 individuals and had passed ethical approval issued by The Ethics Committee of the Faculty of Medicine, University of Indonesia, number 104/UN2.F1/ETIK/2017. Materials used were 96 wells ELISA plate (Nunck), PBS 0.1 M pH 7.4, dH_2O, BSA 10%, anti-blood group A antigen monoclonal antibody (ThermoFisher Scientific), anti-blood group B antigen monoclonal antibody (ThermoFisher Scientific), goat anti-mouse IgG (antibody) peroxide conjugated (Thermo Scientific), TMB (tetramethylbenzidine) and H_2SO_4 0.1 M.

Methods

About 100 mg of mucus from lumen of human intestine was first taken and homogenized in 1 ml of PBS 0.1 M pH 7.4. Then the samples were centrifuged 3000 rpm at room temperature for 10 minutes. The supernatants were taken to be used in ELISA. To coat the microplate well surfaces with antigens, 100 µl of diluted samples were applied in duplicate and incubated in several period and temperature. At the end of the incubation time, the wells were washed three times by addition of 200 µl PBS-Tween 20, incubated 2 minutes in room temperature and followed by blocking, which were performed by applying 100 µl BSA to each well and incubated for 2 hours at room temperature. The wells were washed three times with the same washing solution and in the same manner. After removing the last wash solution, antigen detection was done by pipetting primary antibodies, i.e anti-blood group A or B antigens monoclonal antibodies.

Primary antibodies anti-A and anti-B were used after dilution 1000 times with PBS. Diluted primary antibodies A and B were applied 100 µl and incubated overnight at 4°C. After the period of incubation, the excess of primary antibodies was discarded and the wells were washed three times with the same washing buffer as previously described. Prior to use, secondary antibody was diluted 5000x with PBS. Diluted secondary antibody was applied 100 µl to each well and incubated for 2 hours at room temperature. After incubation, the secondary antibody was discarded and the wells were washed as described earlier.

Enzymes detection, thus indicate the presence of immune complex and hence the antigen was done by applying TMB substrate 100 µl per well and incubated 30 minutes in the dark at room temperature. After 30 minutes, the enzymatic reaction was stopped using H_2SO_4 0.1 M.

The results show yellow colours in various density and are photographed. As this is an optimization of the use of ELISA, the data were all descriptive. The colour changes are only observed and recorded.

RESULTS

Incubation times

Because the antibodies used for ABO blood group does not conceive for ELISA test but for hemagglutination test, the incubation times have to be determined. The results are presented in Table 1.

Table 1. Effect of incubation time of intestinal mucous fixation

Incubation times	Colours
2 hours in room temperature	(-)
Overnight in 0-4°C	(+)

From Table 1, it can be seen that the fixation of intestinal mucous ABO blood groups substance on well surface is a long time process. Table 1 shows that the absorption of intestinal mucous ABO antigens occurred overnight in cold temperature. The results in this study showed that blood groups can be detected from the lumen of human intestines. In the wells that contain the samples, the blood groups were detected, indicated by the yellow colour.

Antibodies anti-ABO maximal dilution

The antibodies anti-ABO used in this experiment were originally conceived for hemagglutination reaction. Therefore,

the dilution recommended by the producer is for the hemagglutination, which is not necessarily proper another immunochemistry reaction such ELISA. For this reason, the maximal dilution for ELISA should be determined.

A series of antibodies dilutions in PBS-Tween 20 were made, starting from 100, 200, 300, 500, and 1000 times. Then 100 µl of each dilution were added into wells which had previously adsorbed the intestinal mucous fluid. The results are presented in Table 2.

Table 2. **The dilutions of primary anti ABO blood group antibodies and colour formation**

The dilution of primary anti ABO antibodies		Colour formation
100 times	Anti A	(+)
	Anti B	(+)
200 times	Anti A	(+)
	Anti B	(+)
300 times	Anti A	(+)
	Anti B	(+)
500 times	Anti A	(+)
	Anti B	(+)
1000 times	Anti A	(+)
	Anti B	(+)

It can be seen that the concentrations of antibodies are very high. After 1000 times dilution, there are still sufficient antibodies which are indicated by the presence of colour formation.

Colour formation

After the addition of TMB, the mixture was incubated in room temperature. The microplate was protected against light by covering with aluminum foil. After 30 minutes, the blue colour appeared and enzymatic reaction was stopped by pipetting 100 µl H_2SO_4 0,1 M. At this moment, the blue colour disappeared, replaced by yellow colour. Meanwhile, in the control wells the dH_2O was used instead of intestinal mucous fluids and there was no colour formation (Figure 1).

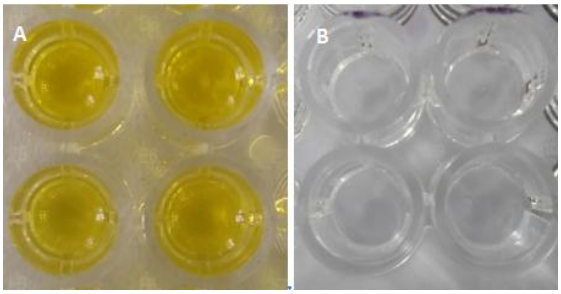

Figure 1. ELISA results. (A) Result of ELISA conducted at human intestines to detect blood group. The left wells are of the blood group A, and the right wells are of the blood group B. (B) In the wells that contains negative controls, ELISA does not show any colours

The picture above is the result of ELISA conducted at human intestines samples and negative control to ensure that the reaction on the samples is not false positive. The negative control wells are not showing any colours because the lumen samples are substituted with dH_2O.

Figure 2 shows the colour formation of the primary anti-A antibody in several dilutions. The dilutions used are 100, 200, 300, 500, and 1000 times. Colours are still formed in 1000 times dilution, although not as clear as the lower dilutions.

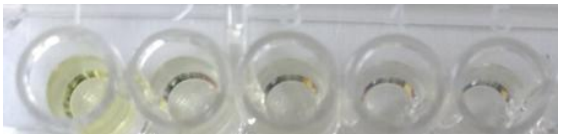

Figure 2. Primary anti A antibody dilutions

The primary anti-B antibody was also diluted 100, 200, 300, 500, and 1000. It also showed colour formation at 1000 times dilution (Figure 3).

Figure 3. Primary anti B antibody dilutions

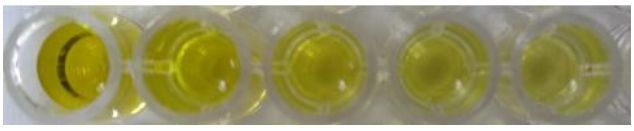

Figure 4. Secondary antibody dilutions

The secondary antibody was diluted 1000, 2000, 3000, 4000, and 5000 times. Figure 4 shows that at 5000 times dilution colour formation still showed and this dilution can be used for further studies.

DISCUSSION

In early 60's detection of ABO blood group from dyed blood smear was developed in forensic studies using agglutination of blood group antibodies[5] and it becomes the standard in determining blood group from blood samples. Anti-ABO hemagglutinin was first analyzed in external fluid in as early as 1928, using saliva as a sample. The technique used was hemagglutination.[4] But, due to insufficient available sample volume, it was not used in legal cases and forensics. In later years, the technique was refined to get 100% accuracy in determining blood group from external body fluid. Normally, detection of ABO blood groups in external body fluid is done by inhibition hemagglutination technique[2,6] or absorption inhibition method.[4] However, this technique still requires a relatively large volume of external body fluid. In the last step of reaction, it needs a standard of red blood cells of A and B groups respectively, to demonstrate that the detecting anti-A or anti-B antibodies are verily bound by A or B blood group substances and then are not anymore available for agglutinating A or B red blood cells.

A very sensitive and specific technique to detect a protein in trace amount using an antibody, labeled with an enzyme, was first developed in mid 60's.[7] Then, in the early 70's, this technique was further developed by using peroxidase-labeled antibody. With this technique, it was possible to measure rat IgG quantitatively with reproducible results.[8] The use of horseradish peroxidase (HRP) as a standard conjugated enzyme was established by van Weemen in 1971.[9] Due to the use of enzyme that is conjugated (linked) to antibody, this technique is called *Enzyme-linked immunosorbent assay* (ELISA). This technique is said to offer more advantages and more secure than its predecessor the radioimmunosorbent techniques (RIST).[10]

In this experiment, detection of ABO blood group in external body fluid was conducted with an in house assembled ELISA method. The ELISA reagents were not obtained as a special kit for this objective but instead were assembled from antibodies anti-ABO for blood group determination and a second antibody which originated from another experiment. As external body fluid, it was used human mucosal fluid. Each sample was detected for blood group A and B. For detection blood group, it is needed only 100 µl of diluted sample to coat the microplate. In total, the ELISA method only needs about 200 µl of solution to perform. The samples that act as the antigens are coated to polystyrene surface of the microplate.

To ensure that the samples are bound to the microplate surface, the samples are incubated in the microplate. Initially, the duration of incubation was 2 hours at room temperature, but it binds poorly. It did not yield result i.e. did not show any colour. It was found that the mucosal fluid bound very well after an overnight incubation which was indicated by colour formation. The longer incubation period of antigen is crucial to the result of ELISA. Longer incubation time means that more time for the antigen to bind to the surface of the microplate, ensuring it to be detected by the antibody. It should be remembered that the surface of microplate's well is hydrophobic, whereas ABO substances are hydrophilic compound, which needs a relatively long time to orient their hydrophobic side for interacting easily with a hydrophobic surface.

The lowest anti-ABO antibodies concentration, as well as second enzyme, labeled antibody used for detection. For these objectives, the first antibodies anti-ABO group substances and the second enzyme-labeled antibody were mixed with PBS to obtain various dilutions. The first anti-ABO antibodies were diluted 100, 200, 300, 500 and 1000 times, whereas the second antibody was diluted 1000, 2000, 3000, 4000 and 5000 times with the same buffer. It was found that the anti-ABO antibodies still recognize their specific antigens after 1000 times dilution, and the second antibody still recognizes its targets, the first antibodies even in much higher dilution, i.e 5000 times.

The advantage of ELISA, compared to other labeled immunoassay like RIA (radioimmunoassay) or RIST, FIA (fluoro immunoassay) is that ELISA can be used even without the aid of any instrument such as spectrophotometer. In a qualitative or a semiquantitative assay, the result of ELISA is very clear, depending on chromogenic substrate use, which in turn, depend on the labeling enzyme. Various enzymes were used at the beginning of development of ELISA, which finally focused on two enzymes, alkaline phosphatase and horseradish peroxidase with the predominate in the later. The chromogen is a peroxidation reaction acts as a hydrogen donor for H_2O_2, the substrate of peroxidases. Several chromogens can be used, most of them are phenolic compound. Among them, benzidine is a very sensitive chromogen. Unfortunately, this compound is known also as a carcinogen.[11] TMB, one of the derivates of benzidine, was proven as nonmutagenic in Ames test.[12] In earlier study, TMB was also safer and noncarcinogenic in animal.[13]

This experiment shows that an ELISA test can be performed using an in-house ELISA kit made by assembling reagents exist currently in laboratories. In this study, first anti-ABO antibodies were originally made for agglutination of human erythrocyte, in which the ABO oligosaccharides are bound to glycosphingolipid of erythrocytes membrane, whereas in mucosal secretion of secretor are bound to a mucosal apoprotein.[14] The difference of anchoring molecules for the same ABO oligosaccharide could result in kinetic of binding, which was expressed time of antibody-antigen reaction. The reaction occurs in relatively long time, whereas in original agglutination the same antibodies recognize, bind and precipitate the erythrocyte containing ABO oligosaccharide immediately. The second antibody or revealing antibody, using in this experiment, is part of another ELISA kit, not specifically for blood group determination. However, as the revealing antibody is made specific to mouse IgG, it

can recognize and bind any mouse IgG everywhere.

CONCLUSION

ABO blood group can be detected in external body fluid using ELISA (technique using antibodies not specifically conceived for ELISA provided that the antibodies are specific for the targets.) The advantage of this technique over other methods is that it can use small volume of sample. The method is relatively easy to do and does not require advanced equipment.

REFERENCES

1. Watkins W. The ABO blood group system: historical background. Transfusion medicine. 2001;11(4):243–265.

2. Mohn J, Owens N, Plunkett R. The inhibitory properties of group A and B non-secretor saliva. Immunological communications. 1981;10(3):315–340.

3. Saboor M, Ullah A, Qamar K, Mir A, et al. Frequency of ABH secretors and non secretors: A cross sectional study in Karachi. Pakistan journal of medical sciences. 2014;30(1):189.

4. Metgud R, Khajuria N, Mamta GR. Evaluation of the secretor status of ABO blood group antigens in saliva among southern Rajasthan population using absorption inhibition method. Journal of clinical and diagnostic research: JCDR. 2016;10(2):ZC01.

5. Kind S. Absorption–elution grouping of dried blood smears. Nature. 1960;185(4710):397.

6. Boyd WC, Shapleigh E. Separation of individuals of any blood group into secretors and non-secretors by use of a plant agglutinin (lectin). Blood. 1954;9(12):1195–1198.

7. Avrameas A. Methode de marquage d'antigen et danticorps avec des enzymes et son application en immunodiffusion. Cr Acad Sci, D. 1966;262:2543–2545.

8. Avrameas S, Guilbert B. A method for quantitative determination of cellular immunoglobulins by enzyme-labeled antibodies. European journal of immunology. 1971;1(5):394–396.

9. Van WB, Schuurs A. Immunoassay using antigen—enzyme conjugates. FEBS letters. 1971;15(3):232–236.

10. Engvall E, Perlmann P. Enzyme-linked immunosorbent assay (ELISA) quantitative assay of immunoglobulin G. Immunochemistry. 1971;8(9):871–874.

11. Baan R, Straif K, Grosse Y, Secretan B, El GF, Bouvard V, Benbrahim-Tallaa L, Cogliano V. Carcinogenic of some aromatic amines, organic dyes, and related exposure. The Lancet Oncology. 2009;9(4):322-323

12. Chung KT, Chen SC, Wong TY, Li YS, Wei CL, Chou MW. Mutagenicity studies of benzidine and its analogs: Structure-Activity Relationship. Toxicological Sciences. 2000;56(2):351-356

13. Holland VR, Saunders BC, Rose FL, Walpole AL. A safer subsititute for benzidine in the detection of blood. Tetrahedron. 1974;30(18):3299-3302.

14. Watkins WM. Genetic and biochemistry of some human blood groups. Proceedings of the Royal Society of London. Series B. Biological Sciences. 1978;202(1146):31-35.