Optimization of Polyethylene Glycol Concentration as an Agglutination Potentiator for Examination of Blood Types of Dry Blood Sample

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Abstract. Blood type is classifying blood based on the presence of antigens on the surface of the erythrocyte membrane. In criminal cases, dry blood spots are often used as samples in determining blood types an the examination with the elution absorption method but this method requires a long and complicated time. polyethylene glycol (PEG) has potentiator properties in the formation of agglutination. The purpose was to determine the optimum concentration of PEG of 1%, 2%, 3%, 4%, 5% as an agglutination potentiator. The experimental research method is blood sample dried on wool yarn overnight. the analysis results obtained on the elution absorption method and without PEG as a control is grade agglutination +2, whereas with the addition PEG 5%, 4%, 3%, 2%, 1% obtained grade agglutination +4, +4, +3, +3, +3 results. The results statistic showed that there was quality of the results of agglutination grade were different at each additional concentration of PEG. At the addition of 4% PEG gave optimal results. So that the PEG modification method can be used as a agglutination potentiator in the determination of blood groups of dried blood spots.

1. Introduction

The ABO system blood type is an indicator of a person’s identity. In living people, blood groups are often used for the benefit of transfusions and donors, while in people who have died the use of blood groups is more focused on forensic identification. In a number of criminal and non-criminal cases, for example disputed parentage, blood type can be a clue to one's identity [1]. In some cases of death with evidence in the form of blood stains on the body, identification of this blood type is very important in relation to the suitability of the blood type on the evidence because the blood type provides specific identity data.

On examination at the crime scene (TKP), blood spots are not always always found fresh, often have dried up, as well as blood stains found on the victim's clothing for example sexual crime, so to determine the blood type requires more difficult techniques and procedures because blood stains that have stuck to the fabric fibers of the victim's clothing wear

Forensic cases that are often asked for blood tests are dried blood). Determination of blood groups in blood spots that are already dry is still possible because the antigens on the surface of the cell remain intact even though the cells have been destroyed, with certain tests these antigens can be reacted with antibodies so that blood groups can still be determined. determination of blood type for
spotting of dried blood can be done by conventional methods of elution absorption, but this procedure is quite difficult because it requires skills with certain techniques, the examination process takes a long time and is usually only done in laboratories with adequate facilities.[2]

The principle of the elution absorption method is; blood samples containing antigens and antibodies, anti-A and anti-B serum added, the added antibodies will bind to the specific antigen, the excess antibodies will then be removed through the washing process, so that what remains is only a specific antigen-antibody bond, then the antibodies will be separated from the antigen because it has eluted in the presence of heating, so that the eluate of red blood cells that have been identified by their blood type because the antisera serum has been dropped. Agglutination can also occur if the added blood cells are the same as the original blood type found in dried blood stains because they have the same antigens on the surface of the same blood cells [3]

So it is necessary to do research to find alternative examination procedures in order to facilitate the examination of blood groups on blood spots that are already dry. The method is to accelerate the antigen in the dried blood spots and the antibodies found in the antisera that will react to form agglutination by adding a potentiator.To increase agglutination and speed up the incubation time in blood type examination, then the agglutination potentiator is used. Potentiator is a reagent that increases the sensitivity of an antigen, one of which is polyethylene glycol .[4]

Polyethylene glycol is a proven potentiator that detects the reaction of polyethylene glycol antigens and antibodies better than polybrene and low ionic strength saline (LISS) because it can detect weaker antibody reactions better, accelerate incubation time, and lower false positive rates. The use of polyethylene glycol has been studied that polyethylene glycol 4000 with a concentration of 20% can detect weaker reactions between antigens and antibodies better than other potentiators such as LISS and polybrene.

Some researchers have compared the use of PEG and LISS which are included in the reaction medium that can be used in antiglobulin tests. Examination results report that polyethylene glycol can increase antibody detection.

2. Research Method

This research is experimental in this research that is blood spots that have been soaked with 0.9% NaCl solution volume 6 ml (3/4 tube), then the blood group is examined with a modified method of adding PEG. Then the blood spots sample added by PEG made a treatment group then observed the agglutination formed and compared with the formation of agglutination in the absorption-elution method which is the control group.

3. Experiment Unit

The sample of this study is that individual blood groups consist of blood groups A, B, AB, and O which are dried on wool yarn media into spots of dried blood each one sample.

4. Tools and Materials

Glass objects, dropper pipettes, micropipettes, yellow tips, 3 ml syringes, test tubes, test tube racks, petri dishes, timers, fins, refrigerators, centrifuges, waterbaths, microscopes ABO system blood group examination reagents, 0.9% NaCl, Phosphate Buffer Saline (PBS), Polyethylene Glycol (PEG) 6000, methanol, wool yarn.

5. Blood type examination

5.1 The Absorbance-Elution Method
- Wool yarn containing spotting of dried blood is fixed with methanol for 15 minutes
- The yarn is removed, allowed to dry, then broken down into fine fibers
- Also carried out on threads that do not contain blood spots as a negative control
Fiber yarn is inserted in two test tubes
Drop anti-A serum into the first tube and anti-B in the second tube
The tubes are stored in a refrigerator at 4°C for one night
The solution is washed with cold 0.9% NaCl solution (4°C) 5-6 times
The test tube is placed in a water bath at 56°C for 10 minutes to elute the antibody and transferred the eluate to another tube
Drop 0.5% suspension of red blood cells A and B into each test tube
One drop of solution is placed on a glass object and examined for agglutination using a microscope at 40x magnification.

5.2 Modification Method
One wool thread containing 20 µl bloodstains is put into a test tube (done for all blood groups)
Add 0.9% NaCl solution as much as ¾ test tube and leave it soaked until the wool yarn does not contain blood spots, then remove the wool yarn using sterile tweezers (± 15 minutes)
Wash cells 3 times by centrifuge at a speed of 3000 rpm for 2 minutes then separate the blood cells from the supernatant
Blood cell samples are made into a 5% suspension
Add one drop each as follows:
- Tube I: Anti-A + 5% cell suspension + PEG 5% / 4% / 3% / 2% / 1% / without PEG
- Tube II: Anti-B + 5% cell suspension + 5% PEG / 4% / 3% / 2% / 1% / without PEG
Shake slowly so that it is homogeneous
Centrifuge with a speed of 3000 rpm for 15 seconds
The tube is shaken slowly and seen with agglutination macroscopically, then calculate the formation time of agglutination.

5.3 Interpretation of Results
Assessment of agglutination that is formed is seen by:
+4: All sediments unite, clear liquid
+3: Sediment split or several with lumps big
+2: The lump is more rough
+1: Very large lumps and smooth, murky liquid looks reddish in color
±: Agglutination such as positive 1 at a glance still looks like a soft lump with a reddish background or agglutination is only seen microscopically
-: There is no agglutination

6. Research Results
In this study a modified blood type examination was performed on a dried blood spot sample with the addition of polyethylene glycol (5%, 4%, 3%, 2%, 1%, and without the use of PEG), as a control the blood group examination was carried out by elution absorption method. The variables observed in this study, namely the level of agglutination grade and the time of formation of agglutination on the examination of blood groups A, B, AB, and O. After observing the results obtained grade agglutination as shown in the table:
Table 1. Observation Results Grade agglutination

| Repetition of Blood Type | Absorbsi Elusi methode | Metode Modifikasi PEG |
|--------------------------|-------------------------|-----------------------|
|                          | Without PEG             | 5%   | 4%   | 3%   | 2%   | 1%   |
| A.1                      | +2                      | +2   | +4   | +4   | +3   | +3   |
| A.2                      | +2                      | +2   | +4   | +4   | +3   | +3   |
| A.3                      | +2                      | +2   | +4   | +4   | +3   | +3   |
| A.4                      | +2                      | +2   | +4   | +4   | +3   | +3   |
| A.5                      | +2                      | +2   | +4   | +4   | +3   | +3   |
| B.1                      | +2                      | +2   | +4   | +4   | +3   | +3   |
| B.2                      | +2                      | +2   | +4   | +4   | +3   | +3   |
| B.3                      | +2                      | +2   | +4   | +4   | +3   | +3   |
| B.4                      | +2                      | +2   | +4   | +4   | +3   | +3   |
| B.5                      | +2                      | +2   | +4   | +4   | +3   | +3   |
| AB.1                     | +2                      | +2   | +4   | +4   | +3   | +3   |
| AB.2                     | +2                      | +2   | +4   | +4   | +3   | +3   |
| AB.3                     | +2                      | +2   | +4   | +4   | +3   | +3   |
| AB.4                     | +2                      | +2   | +4   | +4   | +3   | +3   |
| AB.5                     | +2                      | +2   | +4   | +4   | +3   | +3   |
| O.1                      | -                       | -    | -    | -    | -    | -    |
| O.2                      | -                       | -    | -    | -    | -    | -    |
| O.3                      | -                       | -    | -    | -    | -    | -    |
| O.4                      | -                       | -    | -    | -    | -    | -    |
| O.5                      | -                       | -    | -    | -    | -    | -    |

The observations show that the agglutination grade in the modification method can be observed more clearly than the elution absorption method. The agglutination grade in the modification method shows +4, and +3 good, and without using PEG the results are the same as the standard, which is the elution absorption method, but this method requires a working time.

Figure 1. Result Absorbsi Elusi Methode

Figure 2. Modifikasi PEG 5%
**Figure 3.** Modifikasi PEG 4%

**Figure 4.** Modifikasi PEG 3%

**Figure 5.** Modifikasi PEG 2%

**Figure 6.** Modifikasi PEG 1%

**Figure 7.** Without PEG
Table 2. Means Time of Formation of Agglutination Methods Modifications with variations in PEG concentration

| Blood Type | Without PEG | Modification Methods |
|------------|-------------|----------------------|
|            |             | 5%  | 4%  | 3%  | 2%  | 1%  |
| A          | 15”         | 2”  | 2”  | 5”  | 8”  | 10” |
| B          | 15”         | 2”  | 3”  | 5”  | 7”  | 9”  |
| AB         | 16”         | 2”  | 3”  | 5”  | 7”  | 10” |

Note: ”= seconds.

The observations of the average agglutination time at its peak in each concentration showed that the addition of PEG with concentrations of 1%, 2%, 3%, 4%, 5% showed agglutination formed at the 10th second. Whereas without the addition of PEG shows agglutination appears after 15 seconds. That indicates that the agglutination process is formed more quickly by the addition of PEG.

Blood type O does not count when agglutination because blood type O does not have antigens so that agglutination or agglutination does not occur negative / (-) and for this reason the data for observation of blood type O is not processed statistically. Observational data that were processed statistically were blood types A, B, and AB.

Figure 8. Means Graph of Incubation Time

By considering the time of agglutination formation, the 5% and 4% PEG methods provide shorter time than other methods with the average time of agglutination formation for (2 seconds) and (3 seconds), at concentrations of 3%, 2%, 1% are still formed positive but the formation of agglutination is longer (5 seconds, 8 seconds, 10 seconds) so it is less sensitive to be the optimum concentration in the examination of blood groups instead of the elution absorption method, without PEG giving a significantly longer average agglutination time difference (15 seconds) treatment (other modifications). Based on the results of processing and analysis, it can be concluded that the results of agglutination time are better and faster using a 5% PEG, and the optimum concentration is at a 4%
PEG concentration because the agglutination levels formed are strongly positive with a short time so that it can be used in the examination of alternative blood groups instead the conventional method of elution abortion.

7. Discussion

The mechanism of PEG as a precipitation agent that is negative ions from PEG will combine with the protein which is in the condition as a cation (pH of the solution in acidic conditions to the isoelectric pH of the protein) to form a protein salt. Some of the salts produced are not soluble, so this method can be used to separate the protein from the solution. Generally, the precipitation agent will dissolve while the protein salt will be composed by the addition of a base (forming a negatively charged protein or anionic protein). PEG is generally used for proteins that are already free in blood filtrate and biological fluid testing. PEG is a compound that is stable and soluble in water and functions as a bridge between lipophilic substances with biological fluids that are hydrophilic so that it can unite the mixture consisting of water and fat.

PEG concentration affects the quality of agglutination grade, this is because the size of the molecule and the concentration of PEG in the solution determine the amount of osmotic potential that occurs and is characterized by differences in the grade of agglutination at each concentration of PEG varied. The use of 1-5% PEG can create a better osmotic potential of the solution and can stimulate an increase in the quality of agglutination grade with a higher sensitivity than without the addition of PEG as well as the standard inspection of the elution absorption method, so that the PEG modification method can be used as an indicator of ability PEG compounds in determining the type of blood type. [5]

Conducted research without the addition of PEG is also included in the treatment of the modification method, the results of the agglutination examination did not form a strong positive as in the modification added by PEG. The agglutination grade without PEG is lower than the +4 Likert scale rating criteria which is the best or strong positive rating for agglutination in all blood groups A, B, and AB, so in this study, the formation of agglutination without the addition of PEG is the same as the results formation on the standard that is used as a reference for assessment of +2.

The difference in grade agglutination in the modification method without the addition of PEG can be caused by the red blood cells in the dried blood spots that can be in several conditions, namely: 1). Spots with red blood cells are still intact, 2). Spots with red blood cells have been damaged but with antigens and agglutinins that can still be detected, 3). Red blood cells that have been damaged with the type of antigen that can still be detected but agglutinin damage has occurred, 4). Red blood cells have been damaged with antigens and agglutinins which also cannot be detected. [7]

Therefore, antigens in blood spots that have dried up are difficult to react with antisera blood groups that are added so that in addition to affecting the grade / degree of agglutination that is formed can also affect the time of formation of agglutination as evidenced by the results of the Kruskall Wallis and Mann Whitney U tests where H1 is accepted H0 is rejected, which shows that there are differences in the quality of agglutination and the average value of the formation of agglutination as evidenced in the Graph of Average Incubation Time. [6]

PEG can be used as a potentiator for agglutination and accelerate the incubation time in the modification method because it can detect weak reactions between antigens and antibodies and can accelerate the incubation time because the hydroxyl group from PEG provides a binding site with other molecules. PEG hydroxyl groups can bind water molecules that surround erythrocytes, besides that PEG also acts as a highly mobile molecule between water molecules and other polymers that can leave antigens and antibodies at close range, thus accelerating the clotting reaction.

This research is in accordance with the statement of Fernandes, .kk (2012) in the Journal of Electrical Properties of the Red Blood Cell Membrane and Immunohematological Investigation, which states that one of the potentiators that can be used is polyethylene glycol (PEG) macromolecules. PEG BM 4000 concentration 20% as optimal concentration to increase antigen-antibody reaction in blood type examination [4]. Whereas in this study the concentration of 4% was used to be the optimum concentration. This difference is caused by the type of PEG used in different molecular weights (BM).
PEG 6000 has a molecular weight of 6000-75000 and the number of ethylene oxide group \((n)\) is 136 whereas in PEG has a molecular weight of 3000-3700 and the number of ethylene oxide group \((n)\) is 75.5. In the thesis research according to Azizah (2010) wrote that the estimated interaction of PEG and water occurs through hydrogen bonds between water molecules with ether groups of polymers, so that PEG 6000 can bind more water than PEG 4000.

The ability of PEG to bind water molecules not only impacts the distance of red blood cells, but can also increase the degree of agglutination. The greater the amount of concentration used, the clearer the formation of agglutination formed is compared without adding PEG. A study was conducted without the addition of PEG in the treatment group because they wanted to know and see if PEG was added and what was not added by PEG whether there was a difference or not, the results of the study that showed the difference between adding PEG and not adding PEG proved that the wider distribution of erythrocytes at the bottom of the tube showed the stronger the agglutination is formed, this is because the added PEG causes the antibodies that are scattered in the medium to become concentrated so that the antibodies bind to the antigen which causes the erythrocyte cells to be covered by antibodies in the antisera to form complex antigen-antibody bonds. The binding of antibodies to erythrocyte cells then forms bridges between erythrocyte cells that can be observed as agglutination. The more erythrocyte cells that are connected by antibody antigen groups, the better the grade of agglutination that is formed, in other words strong positive agglutination. So that agglutination in the treatment is stronger than the control group. Theoretically, the control group examination (elution absorption method) has been carried out in stages to increase agglutination when the antibodies are added to the sample and left for a relatively long time, that is, overnight. However, it does not affect the results of agglutination formed better than the treatment group (modification method) but it makes a weakness in this method that is causing a long processing time (2 days) compared to the modification method which only takes 15 minutes of processing time.

Soaking with 0.9% NaCl was carried out by the method of modification of the dried blood spot sample of the treatment group, which was to remove the blood spot sample attached to the wool cloth to dissolve, so that the wool cloth was used only as a medium, and when immersed with 0.9 NaCl % blood patches attached to the wool can be lifted without the remaining blood, then again centrifuged the suspension to separate between NaCl (supernatant) and Blood (sediment) after centrifugation.

Soaking with NaCl solution does not affect the formation of agglutination on blood type examination because the concentration is a physiological or isotonic solution. The isotonic state has ionic concentrations that are similar to the fluid in erythrocyte cells so that it produces the same osmotic pressure. In an isotonic state of fluid that is in the cell, through the cell membrane so that the erythrocyte wall is in normal condition. On the outer wall of erythrocytes there are proteins that are fused with cell walls that are antigenic. Variation in protein in erythrocytes causes blood to be divided into several groups known as blood types. Thus the resistance of erythrocyte cell walls is needed to prevent damage to the blood group antigens that are fused with the erythrocyte cell wall.

The results of the research test between the elution absorption method and the modified method with variations in the concentration of PEG (5%, 4%, 3%, 2%, 1%) and without the addition of PEG showed, there was no difference in the formation of agglutination types of blood types A, B, and AB. Thus the modification method with the addition of PEG can be used as a substitute for the elution absorption method of examining blood groups on dried blood spots, because this modification method does not cause a biased, false negative / positive result on the blood group examination results even though the sample has dried.

8. Conclusion
There are different interpretations of the results of the addition of various variations in the concentration of Polyethylene Glycol as an agglutination potentiator of dried blood spot samples on blood type examination.

The addition of Polyethylene Glycol 4% is the optimum concentration as an agglutination-forming potentiator in the examination of blood group samples of dried blood spots
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