Anti-D quantification by an enzyme-linked antiglobulin test: A comparison study of two methods.
M., Sankian, M., Hashemi, A. R. Varasteh
Immuno-Biochemistry lab, Immunology Research Center, Bu-ali Research Institute, Mashad University of Medical sciences, Bu-ali Sq., Mashad, Iran
Corresponding author: a-varasteh@mums.ac.ir

Abstract
The quantification of anti-red blood cell antibodies routinely performed by standard direct antiglobulin test (DAT), but there are some limits to its sensitivity and the end-point evaluation is very subjective in this method. Recently, a modified enzyme-linked immunosorbant assay (ELISA) has been applied to detect anti-red blood cell antibodies. In this method, the end-point is determined by enzyme activity of an enzyme conjugated anti-human antibodies. The enzyme converts a soluble substrate to a soluble colored product, which is proportional to amount of primary antibodies. This technique has been called enzyme-linked antiglobulin test (ELAT). In this study, we tried to evaluate the analytical parameters of this technique and compare with conventional DAT method. Our results showed some modification of the previously described ELAT technique makes it feasible and simpler for the routine applications. This modified method was reproducible with the mean coefficient variation of 9.08 and 5.21 for between days and within day assays and found to be at least sixteen times more sensitive than DAT method. Anti-D measurement showed an acceptable correlation (R 0.88) between ELAT and DAT methods. Therefore, this method could be useful for more precise monitoring of allo-immunized mothers and patients with autoimmune hemolytic anti-D anemia and provide an alternative method for assessing anti-D activity of specific total IgG and IgG subclasses preparation.
Keyword: ELAT, Anti-Rho(D), DAT.

Introduction
Measurement of anti-D concentration is important, both for anti-D immunoglobulin preparations and monitoring allo-immunized pregnant woman (4,3,5). The standard antiglobulin test provides adequate sensitivity for the routine detection of most erythrocyte antibodies and is simple to perform, but there are limits to the sensitivity of this technique when used for detection of autoantibodies in autoimmune diseases (1). In the other hand, the end-point of the DAT test has to be evaluated ocularly, therefore, the result of the same test could be different when estimated by different technician. Since, it makes DAT method very subjective. Many assay have been developed for quantification of anti-erythrocyte antibody, such as automated heamagglutination, RIA, cytometric method and ELISA (1,10,9) but most of them have not been routinely used for this purpose, because of their disadvantages. Enzyme-linked antiglobulin test (ELAT) has several advantages over standard antiglobulin testing. First, the end point is detected spectrophotometrically, thus increasing the precision and allowing quantitative measurements. Second, the sensitivity could be markedly increased. This paper describes a modified ELAT in microtubes to quantify red cell-bound anti-D antibodies and its comparison with standard antiglobulin test.

Materials and methods
Red blood cells: human erythrocytes were obtained from healthy group O Rh-positive and Rh-negative (as control) donors in EDTA anticoagulant maintained at 4 °C and

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used within 10 days. Red blood cells were washed four times with saline and one time with phosphate buffered saline containing 0.25% BSA (w/v). This should be freshly prepared. Washed erythrocytes were resuspended to 10% (v/v) concentration in citrate buffer (sodium citrate 0.04 M in sodium chloride 0.09 M pH 6.9) containing 1% BSA (w/v).

Sera and standard curve: Thirteen serum samples containing anti-D antibody collected from allo-immunized mothers obtained from Mashad blood bank, were selected. Standard curve was prepared by using 16 to 1024 serial dilutions of Rho (D) immunoglobulin (RhoGAM) with the concentration of 0.073µg/ml to 9.37µg/ml obtained from Ortho Diagnostics (Raritan, NJ).

Standard direct antiglobulin test: The DAT was accomplished according to the procedure in the American Association of Blood Bank (AABB) technical manual, using monospecific Anti-IgG antiglobulin.

Enzyme-linked antiglobulin test: A 100 µl of 10% red blood cells was transferred to 1.5 ml microtube, which has been treated previously with 3% BSA (w/v) in phosphate buffer for 2-3 h at 37 °C. 100 µl of each dilution of standard Anti-D preparation or patient sera was added to precoated microtube. Serial two-fold dilutions were prepared in citrate buffer (sodium citrate 0.04 M in sodium chloride 0.09M, pH 6.9) containing 1% BSA (w/v). For control, 100 µl of citrate buffer containing 1% BSA (w/v) was added to two other tubes. We performed the same procedures for Rh-negative red blood cells. Microtubes were incubated at 37 °C for 1h with occasional gentle agitation. After this incubating, the tubes were incubated again at 4 °C for 10 minute, the red cells then resuspended in 1.5 ml phosphate saline wash solution (sodium phosphate 0.05 M in sodium chloride 0.9%, pH 7) containing
The absorbance and reducing sensitivity of this procedure. We avoided this problem by incubation anti-human IgG at 4 °C, addition of 1% BSA to all media and dilution of chromogenic substrate with saline.

Fig 1 shows the absorbance value obtained with two fold serial dilution of a 300 µg/ml standard anti-Rho (D) immunoglobulin (RhoGAM) using Rho-positive erythrocytes. The optimum working dilution of conjugate antibody was determined by checkerboard titration using the dilute anti-Rho positive and negative control sera.

Table 1. Reproducibility study of ELAT by assessment of Anti-D activity within day and in different days (between days).

<table>
<thead>
<tr>
<th></th>
<th>C.V. of Low con.</th>
<th>C.V. of Intermediate con.</th>
<th>C.V. of high con.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Between days</td>
<td>9.6</td>
<td>9.58</td>
<td>8.07</td>
</tr>
<tr>
<td>Within day</td>
<td>7.34</td>
<td>5.32</td>
<td>2.99</td>
</tr>
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A correlation analysis was used to measure the association degree between both ELAT and DAT method. Correlation coefficient between these two methods was 0.88. Anti-D activity estimates obtained by ELAT and the conventional DAT are shown in Fig 2. The ELAT was also found to be sixteen times more sensitive in detecting sensitized erythrocytes than DAT. The DAT results became positive with eight times dilution of Anti-Rho (D) Immunoglobulin. By contrast, the ELAT was positive when

Fig 1. Semi-log plot of the absorbance of the erythrocytes sensitized (Rho(D)+ and Rho(D)−) by serial two fold Rho(D) against Anti-D concentration(ng/ml).

Fig 2: Comparison of Anti-D (µg/ml) concentration in the samples estimated by ELAT and DAT.
15%) and flow cytometry method (CV, 10%) (8). We also compared the ELAT test with conventional DAT method. A coefficient correlation value of 0.88 shows a close correlation between the ELAT and DAT methods (Fig 2). In addition, the ELAT absorbance values were found to be in linear correlation with amount of antibody on the targets (fig 1). The same results also reported by Gilman et al (6). In contrast, the DAT results showed the similar value for a wide range of anti-D concentration.

In conclusion, the described techniques could be used to determine the Anti-D concentration with a relative of about 8 percent. It is also may be used to estimate IgG Anti-D subclasses. The data obtained with ELAT were compatible with the conventional DAT. In spite of the length of the needed to perform, the objective evaluation of the end point and the increased sensitivity of the ELAT should prove its superior for use in quantification of Anti-D activity or weakly positive DAT in the alloimmunized patients or Anti-D preparations.

References

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Discussion

The major problem of the ELAT procedures is Hemolysis caused by the long incubation and low ion strength of substrate solutions (7,8). The use of BSA in the wash buffer, 50% saline in substrate solution and fresh blood of men was found to be suitable methods of preventing of Hemolysis (11). Gilman et al. used a Hemolysis blank for each sample and subtracted the absorbance of this blank from the absorbance of the test sample (9), but in our experiences this procedure was not found to be appropriated method of eliminating Hemolysis affects.

Our ELAT procedure follows the basic format described by other studies (7, 11, 6). Although we had many effort to use horse radish-peroxides conjugated anti-human IgG Immunoglobulin but erythrocytes peroxides results in a high absorbance background in the test tubes. Next, we performed ELAT procedures according to Hirvonen’s ELAT methods (7) but after several experiments, we did not obtain reproducible absorbance values. Finally, we achieved some modification in incubation time of alkaline phoshatase conjugated anti-human IgG and reaction buffers. After, these slight modifications and optimizing of condition for this test, intra-assay and inter-assay showed significant improvement in the CV of replications (Table 1).

It was necessary to analyze the reproducibility of results obtained in the ELAT techniques in order to establish it as a valide technique. The mean inter-assay and intra-assay CV (respectively, 9.08 and 5.21) indicate obvious evidences of the reproducibility of method (Table 1). Our finding was near to Ruiz et al report on CV obtained with the ELAT in tubes (11). Our experiments showed more reproducibility than automated hemagglutination (CV,
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subclass determinations explain the correlation with functional assay results. Vox Sang. 68: 169-76.