The Donath-Landsteiner (DL) test is a serologic test used to detect the presence of a biphasic hemolysin. This autoantibody is seen in patients with paroxysmal cold hemoglobinuria. The test relies on the characteristic cold binding of an IgG autoantibody with specificity to the P blood group antigen. This autoantibody causes complement-mediated red blood cell (RBC) lysis when warmed to body temperature. In this review, we describe the various methods for performing the DL test—namely a direct test, an indirect test, an indirect test with modifications such as the use of enzyme-treated RBCs and two stages, and an indirect antiglobulin DL test—and highlight the advantages and disadvantages of each. Our focus is on the indirect testing method as it is most commonly used in blood bank laboratories. *Immunohematology* 2019;35:3–6.

**Key Words:** Donath-Landsteiner test, paroxysmal cold hemoglobinuria, biphasic hemolysin, hemolysis, autoantibody

The Donath-Landsteiner (DL) test is a serologic test used to detect the presence of a biphasic hemolysin, seen in patients with paroxysmal cold hemoglobinuria (PCH). The test relies on the characteristic cold binding of an IgG autoantibody with specificity to the P blood group antigen, which causes complement-mediated red blood cell (RBC) lysis when warmed to body temperature. Julius Donath and Karl Landsteiner first described the antibody responsible for this hemolysis in 1904. DL antibodies are usually low titer (≤32) autoantibodies that have low thermal amplitude (≤20°C). The biphasic hemolysis test was the first noted for the identification of this autoantibody.

PCH is a rare form of direct antiglobulin test (DAT)-positive autoimmune hemolytic anemia. According to Dacie, there are three types of PCH: acute, chronic syphilitic, and chronic non-syphilitic. Acute PCH occurs primarily in children. Chronic syphilitic and non-syphilitic types can be more difficult to diagnose and are often seen in adult patients. The DL test is the diagnostic test for PCH.

**Principle**

The DL test exploits the biphasic nature of the DL autoantibody, which is usually IgG. Some case reports, however, show these antibodies having IgM and IgA class specificities. Initially, at cold temperatures, the antibodies bind to RBCs, causing complement (C3) to irreversibly bind to the RBCs at the same time. As this complex is warmed to 37°C, activation of the complement cascade leads to intravascular hemolysis of RBCs when the autoantibody dissociates. This test shows a positive result when hemolysis is visibly seen in the test system (Fig. 1).
Indications

The DL test should be considered when a patient presents with symptoms including recurrent fevers, shaking chills, abdominal pain, and laboratory findings of an intravascular hemolytic anemia with both hemoglobinemia and hemoglobinuria. The onset of the disease is predominantly precipitated by a recent upper respiratory illness, either viral or bacterial. The patient’s RBC sample should also have a positive DAT due to C3 only and no demonstrable autoantibody activity by routine methods.

Procedure

The DL test was developed on the characteristic biphasic hemolysis that was seen in vivo when PCH was highly associated with syphilis, and hemoglobinemia was directly related to exposure to cold. The basic steps of the test are to incubate the patient’s sample at a cold temperature to allow the antibody to attach and then to incubate the sample at body temperature to activate complement and detect the presence of RBC lysis. An important aspect of the test is that the patient's samples have to be kept at 37°C after collection. Historically, the test consisted of two different methods: a direct DL test and an indirect DL test. Modifications such as the indirect DL test with enzyme-treated RBCs, a two-stage test, and an indirect antiglobulin test (IAT) were developed and used in patients strongly suspected of having the DL antibody.

Direct DL Test

The direct DL test is performed by collecting two whole-blood samples into tubes without anticoagulant at 37°C. One sample is incubated at 37°C for 1.5 hours, and the other sample is incubated in melting ice (approximately 0°C) for 1 hour and then at 37°C for 30 minutes. Both samples are centrifuged, and the supernatants are observed for hemolysis. A positive direct DL test occurs when only the sample incubated at 0°C and then at 37°C shows hemolysis. This test is primarily used as an initial screening test in hospitals. When this test is negative, the indirect DL test is performed.

Indirect DL Test

The indirect DL test was developed to increase the sensitivity and specificity of the test system. This test is performed on a fresh blood sample that is collected and allowed to clot at 37°C. The clotted sample is then centrifuged.
at 37°C, and the serum is separated from the RBCs. Freshly collected pooled normal sera are used as a complement source.

Label three sets of three tubes: 1A, 1B, 1C; 2A, 2B, 2C; and 3A, 3B, 3C (Fig. 1). Add 10 drops of the patient's separated serum to each of the tubes labeled 1A, 1B, 1C and 2A, 2B, 2C. Add 10 drops of normal pooled sera to each of the tubes labeled 2A, 2B, 2C and 3A, 3B, 3C. Add 1 drop of a 50 percent suspension of washed P+ RBCs to all nine tubes. Place the three tubes labeled 1A, 2A, 3A in a bath of melting ice for 30 minutes and then at 37°C for 1 hour. Place the three tubes labeled 1B, 2B, 3B in a bath of melting ice for 90 minutes. The three tubes labeled 1C, 2C, 3C are kept at 37°C for 90 minutes. After the completion of incubation, gently mix the tubes and centrifuge. Examine the supernatant for hemolysis. A positive indirect test occurs when tubes 1A and/or 2A show hemolysis and there is no hemolysis in any of the other tubes. When this test is negative but there is a strong suspicion for PCH, a modified indirect test can be performed. The three modifications include using enzyme-treated reagent RBCs, performing a two-stage test, and testing the DL antibody by the IAT.

After identification of the biphasic antibody, one can further demonstrate the anti-P specificity by repeating the test steps described previously but adding washed ABO-compatible p or Pk RBCs in place of the P+ RBCs. These tubes should have no observable hemolysis, thus confirming the antibody specificity.

**Enzyme-treated indirect DL test**

The use of enzyme-treated reagent O RBCs is one means of increasing the sensitivity of the indirect DL test. Various enzymes can be used, including 1 percent papain. This modified test is performed similarly to the indirect DL test. Treating RBCs with enzymes causes increased exposure of the P antigen on the RBC membrane. This increased sensitivity allowed for detection of low-titer DL antibodies in two patients whose DL tests were only positive when papain-treated P+ RBCs were used.

**Two-stage indirect DL test**

Currently, the two-stage indirect DL test is not often performed in blood bank laboratories. This test is performed by initially incubating the patient's serum at 0°C with group O RBCs. If hemolysis is present, the tube is centrifuged, and the patient's serum is removed and replaced with freshly collected pooled normal sera. This replacement increases the sensitivity of the test because of the additional complement that is present for the second half of the assay while decreasing antibody inhibition.

**Indirect antiglobulin test**

The IAT can also be used for the detection of the DL antibody because the antibody is IgG. The IAT should be performed with anti-IgG reagents after the low temperature incubation.

**Procedure Summary**

The procedural method for the DL test suggested by the AABB is the described indirect DL test. This indirect testing method is widely accepted.

**Limitations**

The DL test has several limitations. The blood bank staff performing the procedure need to be highly skilled and meticulous about the temperature requirements of the DL sample throughout collection, clotting, and testing. Given these restrictions, the procedure requires considerable time and resources and is expensive to perform at institutions with limited resources and expertise. At institutions performing high-complexity testing, this procedure can be simple to perform. The timing of the blood sample collection is also important. The DL antibody is transient, and the in vivo titer rises and falls very quickly. The titer is at its highest concentration during the period of clinical hemolysis.

In addition, there are further limitations that depend on the assay used. The direct DL test is more prone to false-negative results than the indirect DL test. This finding could be due to low antibody titer, low complement level, or C3dg presence on the patient's RBCs. C3dg is protective by preventing complement-mediated lysis. Low complement levels in the patient due to consumption during the hemolytic process can also cause a false-negative result. Also, additional sample volume is needed when performing the direct DL.

The indirect DL test can be falsely negative when the antibody titer is low. False-negative results can also occur because of autoadsorption of antibody (if serum separation is not carried out strictly at 37°C) or neutralization of anti-P by globoside in fresh serum (added as a complement source). This latter scenario can be avoided by performing a two-stage DL test.

The enzyme-treated indirect DL test makes the treated RBCs more prone to lysis and therefore comparison with controls is important. Use of the IAT can lead to a false-positive
result due to carryover of direct agglutination by a cold IgM antibody.\(^1,11\)

Another limitation occurs when the patient’s serum is red (due to free hemoglobin) before the test.\(^1,2\) Preanalytical analysis of hemolysis should be taken into account, and special care should be taken to avoid hemolysis during the collection process. If the patient experiences in vivo hemolysis, the presence of free hemoglobin in the serum may be unavoidable.

Both the direct and indirect DL test can be falsely positive when lysis occurs because of the presence of a cold-reacting IgM autoantibody.\(^2\) This reactivity is usually seen in patients with cold agglutinin disease.

**Quality Control**

The tubes labeled 3A, 3B, 3C serve as a negative control for the indirect DL test. Other negative controls include the tubes incubated only at 37°C (for both direct and indirect tests) and those incubated only in melting ice.

**Summary**

The DL test is the diagnostic assay for detecting the presence of a biphasic hemolysin. A high index of suspicion is required when a patient presents with symptoms and laboratory findings consistent with PCH, given the variable titer of this antibody. The DL test can be performed in several ways: direct, indirect, or indirect with modifications. Each of these assays has benefits and limitations.

**References**


Morgan Kilty, MHA, MLS(ASCP)\(^{\text{CM}}\), SBB, Product Manager, QualTex Laboratories, San Antonio, TX; and Tina S. Ipe, MD, MPH (corresponding author), Medical Director of Donor Services, Associate Medical Director of Transfusion Medicine, Houston Methodist Hospital, Department of Pathology and Genomic Medicine, 6565 Fannin Street, MS 205, Houston, TX 77030, tsipe@houstonmethodist.org.