TEST EVALUATION
Rae A. Joselson, M.D.

TEST

Direct Immunofluorescence Antibody Staining (DFA) for Legionella bacteria

PRINCIPLES OF TEST

The DFA is an immunofluorescence procedure for detection of Legionella bacteria in lung tissue, respiratory tract fluids, and cultures. It may be used on fresh or frozen tissue, formalin-fixed tissue, paraffin-embedded tissue, or lower respiratory tract fluids such as sputum, transtracheal aspirates, bronchial washings, and pleural fluids. It may also be used to stain culture smears.

L. pneumophila is a small Gram negative rod that does not grow on routine bacteriologic culture media. The known spectrum of response to infection with L. pneumophila includes asymptomatic seroconversion, a mild self-limited febrile illness characterized by headache, chills and myalgia, and unassociated with pneumonia (Pontiac Fever) and the severe, potentially fatal illness known as Legionnaires' disease, characterized by progressive pneumonia, and sometimes bacteremia. Clinical manifestations caused by more recently identified species of Legionella resemble those due to L. pneumophila infection.

METHOD

Imprints of fresh or frozen tissue, smears of tissue homogenates, lower respiratory tract fluids, formalin suspensions of cultures, or scrapings of formalin-fixed tissue are applied to glass slides. The slides are air dried, heat-fixed and, in the case of fresh or frozen tissue and respiratory tract fluids, fixed in neutral formalin. Deparaffinized slides are used directly. After rinsing in distilled water and air drying, the slides are overlaid with fluorescein isothiocyanate (FITC)-labeled polyvalent conjugates of rabbit antibodies to strains of Legionella. After 20 minutes, excess conjugate is removed, and the slides are immersed in phosphate buffered saline for 10 minutes. This is followed by rinsing in distilled water, air drying, and coverslip application. The stained slides are then examined for morphologically characteristic bacteria, using a fluorescence microscope. The Legionella organisms are observed as brilliantly fluorescent yellow-green rods.

Interpretation of Test Results and Technical Limitations

Specimens are tested using 3 polyvalent pools of antisera. Serotyping of specimens which are positive with a polyvalent reagent can be performed using monovalent reagents. The Centers for Disease Control recommends using the following criteria to evaluate the test results for specimens other than sputum.

<table>
<thead>
<tr>
<th>Result</th>
<th>Report</th>
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<tbody>
<tr>
<td>25 strongly fluorescing bacteria/smear</td>
<td>positive</td>
</tr>
<tr>
<td>&lt; 25 strongly fluorescing bacteria/smear</td>
<td>numbers only</td>
</tr>
<tr>
<td>0 strongly fluorescing bacteria/smear</td>
<td>negative</td>
</tr>
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</table>
With sputum, because organisms are seldom numerous, observation of more than 5 morphologically typical bacteria should be regarded as a positive result. Edelstein et al. consider the presence of 1 to 4 morphologically characteristic fluorescent organisms per smear as positive if at least 2 additional smears demonstrate 1 fluorescent organism per smear. They regard the presence of 5 or more morphologically characteristic fluorescent organisms in any single smear as positive, regardless of the source of the specimen.

No quantitative data is currently available about the precision and accuracy of the DFA. The antisera conjugates have been evaluated with over 374 strains of bacteria and 1 strain of Pseudomonas fluorescens has shown cross reactivity. Other bacteria, cells, and debris may also fluoresce and be misinterpreted as Legionella organisms, producing false positive results. Conversely, Legionella bacteria may disintegrate from the effects of cellular defense mechanisms and exhibit atypical morphology or appear as fluorescent debris. These forms may go unnoticed, potentially leading to false negative results. Such discrepancies may be minimized by the use of counterstains and positive and negative controls, concurrently with the specimen to be examined. Nevertheless, Edelstein et al. found that considerable experience on the part of the observer was required to perform the DFA. New serogroups of L. pneumophila and new species of Legionella organisms are continuing to be identified and these organisms may not react with currently used conjugates.

The sensitivity and specificity of the DFA have been examined in several papers. Edelstein et al. evaluated the reliability of the diagnostic tests for Legionnaires' disease in 32 patients with this infection. They reported the following results for DFA examination.

<table>
<thead>
<tr>
<th></th>
<th>DFA* vs Culture</th>
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<th>DFA* vs Serology (Igm &amp; IgG)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Serogroups I,II,III,IV</td>
<td>Serogroup I</td>
<td>Serogroups I,II,III,IV</td>
</tr>
<tr>
<td>% sensitivity</td>
<td>62</td>
<td>62</td>
<td>43</td>
</tr>
<tr>
<td>% specificity</td>
<td>94</td>
<td>100</td>
<td>88</td>
</tr>
<tr>
<td>% negative predictive accuracy</td>
<td>75</td>
<td>75</td>
<td>66</td>
</tr>
<tr>
<td>% positive predictive accuracy</td>
<td>89</td>
<td>100</td>
<td>75</td>
</tr>
</tbody>
</table>

* DFA of respiratory secretions before therapy only

These authors found lung tissue and sputum to be the optimal specimens for the DFA.

Broome et al. evaluated the specificity of DFA staining in sputum specimens and found no false positives among 47 controls with pneumonia caused by agents other than L. pneumophila. However, sensitivity was poor, with only 24% of 21 patients having a positive DFA and shown to have Legionnaires' disease by cultural or serologic criteria. These investigators speculated that the low proportion of positivity might be due to the absence of Legionnaires' disease bacterium in the
sputum at certain times during the course of the disease, the inadequacy of some of the specimens, or the storage of specimens for months at room temperature which occurred in their study.

More recently, Zuravleff et al. performed a prospective study of 40 cases of Legionnaires' disease using newer more selective culture media. When culture positivity was used as the basis for diagnosis of Legionnaires' disease, the sensitivity of DFA testing was 33%. When seroconversion was the basis for the diagnosis, the sensitivity of the DFA was 47%. No attempt was made to evaluate specificity or predictive value. In discussing the poor sensitivity of the DFA, these authors noted that the yield from DFA examination is directly related to the number of organisms recoverable by cultural methods; thus, the DFA may be negative in an early or mild case of Legionnaires' disease. Treatment with erythromycin can also lead to negative DFA results.

Evaluation of the sensitivity and specificity of the DFA is hampered somewhat by the lack of a "gold standard" for the diagnosis of infection due to Legionella bacteria; the DFA can only be compared with culture and/or serologic methods. In addition, sensitivity and specificity vary with the source of the DFA specimen, and studies reporting results for each specimen type are too limited in number for definitive analysis. Therefore, most investigators recommend that serologic, cultural, and DFA techniques be used in combination for optimal sensitivity and specificity in the diagnosis of Legionella infection.

REFERENCES


TEST EVALUATION
Stephen Darling, M.D.

TEST
Direct Anti-globulin Test (Direct Coombs) (DAT)

METHOD
Polyspecific antihuman globulin is added to the patients' washed red cells, then incubated, and centrifuged. Visible agglutination of the red cells indicates the presence of bound IgG or C3. All positive reactions are further evaluated using monospecific anti-human IgG and anti-human C3d.

CLINICAL APPLICATIONS
The DAT detects antibodies and/or complement on the surface of patients' red cells which were bound in vivo. The DAT is clinically useful in the evaluation of possible immune hemolytic anemia, in the diagnosis of hemolytic disease of the newborn, and in the investigation of transfusion reactions. A positive reaction indicates red cell sensitization which could lead to shortened red cell survival. The pattern of sensitization, i.e., the presence of IgG or complement alone or in combination, gives additional information regarding the method of sensitization. For example, the presence of C3 in the absence of IgG is typically seen in immune complex type sensitization due to drugs (e.g. quinidine), or with cold autoantibodies. Other patterns are less specific.

CLINICAL AND TECHNICAL LIMITATIONS
The major limitation of the DAT is in the interpretation of results. The sensitivity of current DAT's is such that 100-500 molecules of IgG must be bound to each red cell in order to get a positive test result. Occasional patients are encountered with a negative DAT who obviously have shortened red cell survival due to immunologic destruction. In these cases there must be enough IgG coating of the red cells to decrease in vivo survival, but not enough to give a positive DAT. If the test were to be "improved" in order to be 100% sensitive, a significant number of false positives would result since all normal red cells have a small amount of IgG on their surface.

Unfortunately the strength of the agglutination reaction in positives does not correlate with the severity of in vivo hemolysis. In fact, 10-15% of all patients receiving α-methyl dopa for the treatment of hypertension have positive DATs, but only 1% of these demonstrate shortened red cell survival. This test therefore needs to be interpreted in light of the patient's clinical status.

A number of technical pitfalls exist in the performance of the DAT which may lead to false positive or false negative results. False positives may occur if the blood specimen is not collected in EDTA anticoagulant (lavender top tube). This is due to nonspecific coating of red cells by complement which is activated during the process of collection and clot formation. EDTA chelates free Ca²⁺ in the serum and thus prevents this activation and binding of complement. False positives may also occur
in patients with hyperproteinemic states since rouleaux formation may be erroneously interpreted as agglutination. Nonspecific red cell adsorption of serum proteins including immune complexes as well as polyagglutinable red cells, which are occasionally seen in septic patients and under diverse clinical conditions, are other possible sources of a false positive DAT.

Technical problems leading to potential false negative results include incomplete washing of red cells with residual serum neutralizing the added antiserum, the use of outdated reagents, and the inadvertent elution of the coating antibody during the washing steps. All of these technical pitfalls are currently more theoretical than real since appropriate controls and methods are used to avoid or detect the occasional erroneous result.

REFERENCES

