Detection of Bacterial Pathogens in Purulent Clinical Specimens by Immunofluorescence Techniques

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A technique is described which may be used to identify Haemophilus influenzae type b, Streptococcus pneumoniae, group A and B streptococci, and Staphylococcus aureus in smears of clinical specimens.

The etiology of infection often cannot be ascertained by culture when patients have been partially treated with antibiotics. Antigen detection techniques such as counterimmunoelectrophoresis (2), latex agglutination (10), and staphylococcal coagglutination (9) have been developed in the past several years in an attempt to establish diagnosis in such cases. These methods have been used to detect bacterial antigens in infected body fluids, such as cerebrospinal and pleural fluid, as well as in serum and urine when antigenemia is present. However, the techniques may not be applicable for purulent specimens, such as those obtained from a septic joint or an abscess. Deposits of cellular debris, mucus, or fibrin interfere with the precipitation reaction and distort the agar gel in the counterimmunoelectrophoresis procedure. Nonspecific agglutination reactions occur in the presence of purulent material in both the latex and coagglutination assays.

Haemophilus influenzae type b (HIB), Streptococcus pneumoniae, group A and B streptococci, and Staphylococcus aureus are some of the more frequent causes of meningitis, pneumonia, septic arthritis, otitis media, and abscesses in infants and children. This report suggests that an indirect fluorescent-antibody technique may be applied to detect these pathogens in culture-negative specimens. Examination of all five agents may be carried out simultaneously on a single smear of the specimen. Although no specific antiserum is available, detection of S. aureus is possible because rabbit immunoglobulin G strongly binds to protein A in the staphylococcal cell wall (4, 7). The binding of rabbit immunoglobulin G to the organisms is detected by application of fluorescein-conjugated anti-rabbit gamma globulin.

A thin smear of the specimen was made on a glass slide, allowed to dry, and then heat fixed. Parallel marks (approximately 4 mm in length and 2 mm apart) were etched with a diamond pencil in four widely separated areas of the smear. To each area, a small drop of one of four specific antisera was applied. Rabbit antisera to HIB (Hyland Diagnostics, Deerfield, Ill.), S. pneumoniae (Omniserum, Staten Serum Institut, Copenhagen, Denmark), and group A and B streptococci (Wellcome Reagents, Research Triangle Park, N.C.) were routinely used. All primary antisera were diluted 1:64 in phosphate-buffered saline (pH 7.2) before use. Preliminary studies demonstrated that lower dilutions of the sera frequently produced cross-reaction fluorescence, whereas specific activity decreased when sera were diluted beyond 1:64.

The smear was incubated at room temperature in a moist chamber for 30 min. The smear was washed by holding the slide in a tilted position and applying buffered saline from a wash bottle for 10 s. The slide was then placed in buffered saline for 10 min, dipped into tap water, and blotted dry. Fluorescein-conjugated goat anti-rabbit gamma globulin (Antibodies Incorporated, Davis, Calif.) diluted 1:200 in buffered saline was added to all of the etched areas. The incubation and washing procedure was repeated as described above. The smear was mounted in buffered glycerol and examined by fluorescence microscopy. Five smears were prepared from fresh isolates of HIB, S. pneumoniae, group A and B streptococci, and S. aureus. Each smear was stained with all four antisera in the same manner as the clinical specimen to provide positive and negative controls. Identification of HIB and the streptococci was based on the observation of fluorescent organisms with appropriate morphology in one of the four stained areas of the smear, i.e., rods, lancet-shaped diplococci, or very round cells in pairs or chains. S. aureus was identified by fluorescent clusters of cocci observed in all four areas of the smear. Fluorescence staining was reported as positive...
only when fluorescence was as bright as in the positive control smears.

Counterimmunoelectrophoresis was used to detect the polysaccharide antigens of HIB and S. pneumoniae, using the above-mentioned antisera (undiluted). The test was performed with a counterimmunoelectrophoresis supply package (Hyland Diagnostics) according to the manufacturer’s directions.

Smears were selected for examination from 72 specimens which yielded HIB, S. pneumoniae, group A or B streptococci, or S. aureus in culture. Results of fluorescence staining are shown in Table 1. The sources of the specimens and the number of specimens from each source were: cerebrospinal fluid, 30; abscess, 15; joint fluid, 14; pleural fluid, 6; sinus aspirate, 2; middle ear aspirate, 2; lymph node, 2; and peritoneal fluid, 1. A specific pathogen was identified by fluorescence staining in 61 (87%) of the 72 specimens. In comparison, organisms were observed by Gram stain in 50 (73%) of the specimens. In the 11 culture-positive but fluorescence staining-negative specimens, there was sparse growth on culture, indicating few organisms in the specimen. In one culture, a single colony was apparent after 48 h of incubation. In another, the organisms were recovered only from the broth medium after 5 days of incubation. Fluorescence with heterologous as well as homologous antisera occurred in two specimens. Both group A and B streptococcal sera produced bright fluorescence in one smear containing HIB and another containing S. pneumoniae. The correct identification was determined by the morphology of the fluorescing bacteria.

The relevance of the technique lies in the ability to detect pathogens in specimens from patients who have been partially treated with antibiotics and whose cultures are sterile. Accordingly, positive fluorescence staining results were obtained for one of the five pathogens on 18 culture-negative specimens. All patients involved were treated for from less than 1 day to several days before the specimens were obtained. Of the 18 patients, 13 had culture-positive specimens before antibiotic administration. The other five specimens gave antigen-positive results by counterimmunoelectrophoresis. The fluorescence staining tests were in agreement with the pretreatment cultures or counterimmunoelectrophoresis results in all 18 specimens. Organisms were seen in the Gram stain in 10 of the specimens.

Smears from 12 specimens which were culture positive for other organisms were examined. The specimens were obtained from normally sterile sites, and the following number of specimens yielded on culture a single isolate of: alpha-hemolytic streptococci, 2; non-group A or B beta-hemolytic streptococci, 2; Neisseria meningitidis, 4; Staphylococcus epidermidis, 2; Pseudomonas aeruginosa, 1; or Bacteroides fragilis, 1. Organisms were seen in the Gram stain in all of the specimens. False-positive fluorescence was observed in two smears. Group A and B streptococcal antisera reacted with a beta-hemolytic streptococcus subsequently identified as group G. The group A serum also reacted with a strain of N. meningitidis in a joint fluid specimen. However, the organism was interpreted not to be a group A streptococcus on the basis of morphology.

Biegeleisen et al. (1) and Fox et al. (5) used the direct technique to evaluate immunofluorescence in the rapid diagnosis of meningitis caused by HIB, S. pneumoniae, and N. meningitidis. In both studies, the pathogen was identified in 93% of the smears of culture-positive specimens. The value of the technique in the diagnosis of partially treated (culture-negative) meningitis was also demonstrated. In the present study, comparable results were obtained by an indirect fluorescent-antibody method on specimens representing a variety of infectious processes. A unique feature of the method is the ability to detect S. aureus. Currently, there are no techniques available to allow identification of S. aureus in culture-negative specimens. The assay for S. aureus is dependent upon the content of protein A in the cell wall. Forsgren (3) found that protein A was produced by 98.9% of 700 coagulase- and deoxyribonuclease-positive strains of staphylococci. However, the strains varied in the amount of protein A produced. It is possible that strains with a low content of protein A in their cell walls would not be readily detected by this technique.

A number of the specimens tested, such as

### Table 1. Comparison of culture, fluorescent-antibody, and Gram stain results from 72 purulent clinical specimens

<table>
<thead>
<tr>
<th>Organism</th>
<th>No. of isolates</th>
<th>No. positive by fluorescence staining</th>
<th>% Positive</th>
<th>No. of organisms observed by Gram stain</th>
<th>% Positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>HIB</td>
<td>29</td>
<td>25</td>
<td>86</td>
<td>19</td>
<td>66</td>
</tr>
<tr>
<td>S. aureus</td>
<td>24</td>
<td>18</td>
<td>75</td>
<td>16</td>
<td>67</td>
</tr>
<tr>
<td>S. pneumoniae</td>
<td>11</td>
<td>11</td>
<td>100</td>
<td>9</td>
<td>82</td>
</tr>
<tr>
<td>Streptococci</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group A</td>
<td>4</td>
<td>4</td>
<td>100</td>
<td>4</td>
<td>100</td>
</tr>
<tr>
<td>Group B</td>
<td>4</td>
<td>3</td>
<td>75</td>
<td>2</td>
<td>50</td>
</tr>
<tr>
<td>Average</td>
<td></td>
<td></td>
<td>87</td>
<td>73</td>
<td></td>
</tr>
</tbody>
</table>
joint fluid, lymph node, abscess, and sinus and middle ear aspirations, were not suitable for antigen detection procedures such as counterimmunoelectrophoresis because of their purulent nature or insufficient volume. In many cases, unstained smears were all that remained of the specimen after culture and other laboratory examinations were completed.

False-positive fluorescence which occurred in several specimens may have been due to (i) cross-reacting antigens, (ii) insufficient washing, or (iii) merging of two or more primary sera during incubation. False-positive results may be recognized by the morphology of the organisms, as in the case of the group A and B streptococcal sera staining smears containing HIB and S. pneumoniae. Morphological assessment was less reliable in specimens in which only intracellular organisms were found.

Fluorescence techniques are more sensitive in the detection of organisms than the Gram stain. Further, the Gram stain allows only a presumptive identification based on the Gram reaction and cellular morphology. An acridine orange fluorochrome staining method to detect organisms in clinical specimens (6) or in blood culture broth (8) has been described. The method is also superior to the Gram stain in revealing organisms in low densities. The advantage over immunofluorescence methods is that any bacterial species can be detected due to staining of the nucleic acids. Although the indirect fluorescent-antibody assay is limited to a few specific agents, it provides simultaneous detection and identification of pathogens in clinical specimens.

LITERATURE CITED