Neisseria gonorrhoeae Identification in Direct Smears by a Fluorescent Antibody-Counterstain Method

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ABSTRACT

WHITE, Lendell A. (Communicable Disease Center, U.S. Public Health Service, Atlanta, Ga.), and Douglas S. Kellogg, Jr. Neisseria gonorrhoeae identification in direct smears by a fluorescent antibody-counterstain method. Appl. Microbiol. 13:171-174. 1965.—Direct smears from female patients have been considered unreliable for the detection of Neisseria gonorrhoeae by fluorescent antibody (FA) methods because of inadequate background contrast of the fluorescent-stained smears and a scarcity of organisms on the smear. Evans blue dye employed as a counterstain eliminated the nonspecific background staining and increased the reliability of the direct FA procedure. Direct smears demonstrating positive fluorescence were obtained from 86% of a group of culturally positive named female contacts. The FA-counterstain technique is as sensitive as the presently recommended cultural procedures.

Immunofluorescent procedures are widely used for the rapid identification of infectious disease agents in tissues and exudates. Deacon et al. (1959) described an application of the direct fluorescent-antibody (FA) method to the identification of Neisseria gonorrhoeae in exudate smears from males. The difficulty in finding N. gonorrhoeae in direct smears from females required that another technique be employed. Deacon et al. (1960) described the delayed FA technique in which the specimens were incubated on an enrichment medium for 16 to 20 hr before staining with fluorescein-labeled N. gonorrhoeae antiserum. By this method, N. gonorrhoeae was demonstrated in 58% of the specimens from named female contacts, whereas only 24% of the direct smears demonstrated positive fluorescence. Specific fluorescence as observed in direct smears may be reduced or obscured by nonspecific staining of background materials. In attempting to reduce this difficulty with other microorganisms, investigators have employed counterstains such as contrasting fluorescent dyes (Smith, Marshall, and Eveland, 1959), chelated azo dyes (Hall and Hansen, 1962), and Congo red or Evans blue dyes (Nichols and McComb, 1962). This report describes the use of Evans blue dye as a counterstain on direct smears of N. gonorrhoeae. A comparison is made of the results obtained with specimens which were examined by direct FA, FA-counterstain, and cultural procedures.

MATERIALS AND METHODS

Collection of specimens. Using a 1-mm nichrome loop, two smears and a culture were collected from 155 named female contacts of males having gonorrhea. The cervix was cleaned with a cotton pledget, removing all mucous and discharge from the external os. A speculum was used to compress the cervix and massage the endocervical glands by the procedures outlined by the U.S. Public Health Service (1962). The specimens were collected by John H. Tiedemann, Venereal Disease Control Officer, Fulton County Health Department, Atlanta, Ga.

Cultural procedures. The basic culture medium employed was Difeo GC medium base with 1% defined supplement (GCBDS). The defined supplement was a modification of that described by Kellogg et al. (1963), and was composed of: glucose, 40 g; glutamine, 1 g; 0.2% cocarboxylase solution, 1 ml; ferric nitrate, 50 mg; distilled water, 100 ml. With a number of specimens, overgrowth of N. gonorrhoeae by contaminating organisms was eliminated by using antibiotics as recommended by Thayer and Martin (1964) at a final concentration of 10 μg/ml of ristocetin (Abbott Laboratories, North Chicago, Ill.) and 25 units per ml of polymyxin B (Charles Pfizer and Co. Inc., Brooklyn, N.Y.). Antibiotics were obtained through the courtesy of James D. Thayer, Venereal Disease Research Laboratory.

Cultural procedures were varied in an attempt to obtain the greatest number of isolations. Specimens collected from 88 patients were inoculated...
onto petri plates of GCBDS medium, and specimens collected from 37 additional patients were inoculated onto petri plates of GCBDS supplemented with antibiotics. In addition, 60 specimens were collected by placing the inoculum into a medium of the same composition as GCBDS without agar. This broth was used as a sustaining menstruum, and included antibiotics at the concentrations previously mentioned. All cultures were stored under CO₂ (candle jar) at room temperature and delivered within 4 hr to the laboratory. Specimens received in the liquid medium were centrifuged at 1,600 × g for 5 min, resuspended in a few drops of broth without antibiotics and inoculated onto plates of GCBDS. Cultures were examined for colonies of N. gonorrhoeae after 18 to 24 hr of incubation at 35 C under CO₂. N. gonorrhoeae isolates were identified by oxidase and carbohydrate fermentation reactions, Gram stain, and FA methods.

Preparation of direct smears. Specimens collected for smears were spread uniformly with the collection loop within a 6-mm circle etched on a microscope slide (25 by 75 mm). The smears were air-dried and gently heat-fixed. Smears were either stained immediately upon receipt at the laboratory or stored at 5 C until examination.

Fluorescein-labeled N. gonorrhoeae rabbit antisera were prepared as described by Deacon et al. (1959). Conjugates were absorbed with Difco beef bone marrow, tested for nonspecific staining with smears of Aerobacter cloacae, and stored at −20 C. Conjugates were routinely diluted with pooled normal human serum and did not stain Staphylococcus aureus or any member of the genus Neisseria other than N. gonorrhoeae. In performing the direct FA procedure, a drop of conjugate from a capillary pipette (0.04 ml) was spread over each smear, and the smear then incubated in a humid chamber at 35 C for 30 min. The smears were soaked for 10 min in phosphate-buffered saline (pH 7.2), blotted dry, and mounted with a drop of glycerol-saline solution (pH 7.2). Fluorescence and typical cellular morphology were the criteria used to determine the positivity of each specimen.

Evans blue counterstain. Fixed smears were rinsed for 1 min in carbonate buffer (pH 9.0) and gently blotted dry. Fluorescein-labeled N. gonorrhoeae antiserum was added, and the smears were incubated as described previously. After incubation, the smears were soaked for 5 min in phosphate-buffered saline (pH 7.2), and transferred without drying to a 1% aqueous solution of Evans blue for 10 min at room temperature. A final rinse was made for 1 min in two changes of carbonate buffer (pH 9.0). The smears were blotted dry and were mounted with a drop of glycerol-saline solution.

The previously described procedure for the acquisition of material for smears and culture required collecting a minimum of three loopfuls of material from each patient. Occasionally it was difficult to acquire this amount of specimen. Although the distribution of material between smears and culture was randomized, it was believed that the total amount of specimen required and its distribution could contribute to a variance between cultural and FA results. After determining the results of the divided samples, an attempt was made to detect variability due to sample division. Fifty-one additional specimens were collected and examined by the procedures which gave the greatest number of positive divided samples. The specimens were collected by placing the loop of material into a tube containing 1 ml of liquid menstruum without antibiotics and storing in the refrigerator until transferred to the laboratory. The cells were packed by centrifugation at 1,900 × g for 5 min, washed twice with phosphate-buffered saline (pH 7.2), and recenterfuged; the cellular material was then spread uniformly within a 6-mm circle on a microscope slide. These smears were examined by the FA-Evans blue counterstain procedure.

Reagents. The carbonate buffer and glycerol-saline solutions were prepared as described by Cherry, Goldman, and Carski (1960).

Microscopy. Leitz and American Optical fluorescence microscopes were used. The light source consisted of an Osram HBO 200 mercury vapor lamp with a Schott BG-12 exciter filter and a Corning 3-72 barrier filter.

Results

The number of specimens demonstrating positive fluorescence in direct smears by the FA-counterstain procedure equaled or exceeded those detected by cultural methods, and the results of both these techniques were superior to those obtained by the direct method without the counterstain. Isolates of N. gonorrhoeae were obtained from 87 specimens. Of these 87 specimens, positive fluorescence was observed with 86% of the direct smears by the FA-counterstain procedure but with only 66% of the smears when the direct method was employed. Fluorescent staining reactions were observed with direct smears from 25 specimens from which isolations were not obtained. Of these specimens, 11 were stained by the FA-counterstain procedure, 6 were stained by direct FA, and 8 were stained by both procedures. No staining reactions were observed with seven specimens from which N. gonorrhoeae was isolated. Isolations were not obtained and fluorescent staining was not observed with 43 specimens. Positive control smears of N. gonorrhoeae were included and examined with the direct FA and FA-counterstain methods. No deviation in the specific fluorescence was observed.

The FA and culture results are compared in Table 1. N. gonorrhoeae was isolated from 48% of the specimens inoculated directly onto GCBDS medium and from 62% of the specimens inocu-
lated either onto GCBDS with antibiotics or into the liquid sustaining medium. Although there was a significant increase in the number of isolations obtained on the medium supplemented with antibiotics, the growth of *N. gonorrhoeae* on this medium required a slightly longer incubation period.

To ascertain the effects of dividing the specimen into three parts, 51 additional specimens were examined by only the FA-counterstain procedure. Fluorescent staining was observed with 33 or 65% of the specimens. These results are comparable to those of the FA-counterstain procedure presented in Table 1 and indicate that sample division of the specimen had little effect.

**DISCUSSION**

The kind of specimen and the manner in which it is obtained are critical to the successful detection of the anticipated microorganism. The bacteriological loop increases the possibility of a complete transfer to a microscope slide of a specimen composed of a small number of organisms. It is believed that the use of a bacteriological loop for specimen collection and the restricting of the smear area were contributing factors to the number of positive specimens obtained by FA examination.

The advantages of the FA-counterstain procedure are an increased contrast between the stained organisms and the background of the smear and the ease with which organisms covered with exudate may be observed. We obtained 45% positive smears with the direct FA procedure, and 60 to 65% positive smears were obtained when the FA-counterstain procedure was employed. Since controls were included throughout the study, the increase in positive specimens is the result of a greater sensitivity caused by the inhibition of nonspecific staining by the Evans blue counterstain. It was observed with the FA-counterstain that organisms were present within the cellular exudate of the smear and their fluorescence was masked by the nonspecific staining of the cellular material.

The cultural methods for *N. gonorrhoeae* are not entirely successful, and gross contamination of the specimen decreases the probability of a successful isolation. As observed in Table 1, the greatest difference between cultural and FA-counterstain results occurred in the group of specimens giving the least number of isolations. The presence of antibiotics in the cultural medium and the sustaining menstruum of the other groups greatly facilitated the isolation of *N. gonorrhoeae* and increased the percentage of isolations. The FA staining of nonviable as well as viable organisms and the failure to isolate *N. gonorrhoeae* from grossly contaminated specimens may have contributed to the 16% of the specimens giving positive FA reactions but negative culture results.

Evans blue dye was selected because it is easily available to most laboratories and may be prepared without special chemicals or equipment. A 1% solution of Evans blue was preferred in our laboratory; however, dye concentrations ranging from 0.5 to 2.5% were examined. The higher concentrations of dye tend to loosen the smear and obscure specific fluorescence.

The rinsing of stained smears in carbonate buffer (pH 9.0) increased and stabilized the brilliance of the specific fluorescent staining. This greater fluorescence increased the contrast between the stained organisms and the background. Nonspecific background fluorescence was diminished when the counterstain was employed. Adjustment of the glycerol-saline mounting fluid to pH 8.4 helped maintain the intensity of the specific fluorescence. However, interference may occur at this pH from low-intensity nonspecific fluorescence of tissue and white blood cells.

**LITERATURE CITED**


### Table 1. Percentage of *Neisseria gonorrhoeae* specimens positive by culture and smears

<table>
<thead>
<tr>
<th>Medium</th>
<th>Inoculation</th>
<th>No. of specimens</th>
<th>Positive cultures</th>
<th>Positive smears</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>%</td>
<td>FA-counterstain</td>
</tr>
<tr>
<td>GCBDS</td>
<td>Direct</td>
<td>58</td>
<td>48</td>
<td>59</td>
</tr>
<tr>
<td>GCBDS plus antibiotics</td>
<td>Direct</td>
<td>37</td>
<td>62</td>
<td>59</td>
</tr>
<tr>
<td>GCBDS</td>
<td>From broth sustaining medium</td>
<td>60</td>
<td>62</td>
<td>63</td>
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