

Method for Preparing *Neisseria gonorrhoeae* Fluorescent Antibody Conjugate

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THE direct-staining fluorescent antibody (FA) technique has proved useful as a laboratory aid in the identification of *Neisseria gonorrhoeae*, or gonococcus (1-12). An effective method for producing satisfactory fluorescein conjugated antisera to this gonococcus (GC) has been found. The method will aid laboratory workers in preparing, checking, and evaluating the conjugates produced in their laboratories or purchased from a commercial source.

Materials and Methods

The following 10 steps were taken to produce a specific and sensitive fluorescein conjugate to identify *N. gonorrhoeae*.

1. *Isolation and preparation of antigens and antisera.* Urethral specimens were collected from five men with gonorrhea and streaked on plates of Difco GC medium base, single strength, with 1 percent supplement B. After incubation at 35° C. with carbon dioxide (candle extinction) for 18 to 20 hours, the colony types (13), usually type 1 or 2, were observed. From each isolate, type 1 colonies were picked and streaked onto each of five plates to insure purification and adequate growth for the production of antigens. After incubation, as before, the cultures were observed for colony types, which were mostly type 1. All cultures were confirmed by sugar fermentation.

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The growth on the plates from each isolate was harvested in phosphate buffered saline (PBS), pH 7.2, with 3 percent formalin, which consisted of 3 ml. of 37 percent formaldehyde to 100 ml. of PBS (1). The suspension was allowed to stand at room temperature for 1 hour, with frequent mixing, then was washed three times in PBS and adjusted to 10 times McFarland No. 10 (14) in 0.3 percent formalin PBS. The adjusted suspensions of each isolate were then combined in equal proportions for injection into rabbits. Five milliliters of the combined suspension was thoroughly emulsified with 5 ml. of Freund's incomplete adjuvant, and each rabbit was inoculated subcutaneously in multiple sites with the 10-ml. mixture.

Subsequent inoculations were given intravenously with killed suspensions prepared from fresh isolates as described. Each of the five isolates was adjusted to McFarland No. 10 in PBS with 0.3 percent formalin, pooled as before, and injected according to the following schedule.

Inoculation schedule

Day 1.....	Subcutaneous in Freund's incomplete adjuvant: 10 ml. (5 ml. <i>Neisseria gonorrhoeae</i> 10 X McFarland 10 plus 5 ml. adjuvant)
Day 7.....	Intravenous 0.5 ml. of 1 X McFarland 10
Day 10.....	Intravenous 1.0 ml. of 1 X McFarland 10
Day 15.....	Intravenous 2.0 ml. of 1 X McFarland 10
Day 20.....	Intravenous 2.0 ml. of 1 X McFarland 10
Day 25.....	Intravenous 2.0 ml. of 1 X McFarland 10
Days 32-35....	Test bleed; titer should be 1:16 to 1:64

A total of 30 different isolates, five isolates per inoculation, was used in the complete schedule. Rabbit blood was tested at various stages during the schedule, and the direct-slide agglutination method (14) was used to follow the antibody titer. Serums with a titer of 1:16 to 1:64 were considered satisfactory for fluorescein labeling.

2. *Preparation of globulin.* Globulin was prepared from suitable antiserums according to the method described by Cherry and associates (15). The globulin was precipitated with one-half saturated ammonium sulfate and centrifuged. The supernatant was removed, and the precipitate was dissolved in water and dialyzed against 0.85 percent sodium chloride (NaCl).

3. *Preparation of fluorescein-labeled globulin.* The method for preparing the conjugate was modified from the technique described by Cherry and associates (15). Newer labeling techniques have been reported to be satisfactory (16, 17).

After protein was determined by a refractometer, the protein solution was adjusted to 1 percent with 0.85 percent NaCl. One part of 0.5 molar carbonate-bicarbonate buffer, pH 9.0, was added to 9 parts of chilled protein solution; then for each milligram of protein, 0.025 mg. of fluorescein isothiocyanate (isomer 1, crystalline, chromatographically pure; Baltimore Biological Laboratory) was added slowly, with stirring (18).

After the fluorescein was added the solution was stirred constantly at 5° C. for another 4 to 6 hours (18). Finally, free fluorescein was removed from the conjugated globulin solution by passing it through fine Sephadex G-25 (Pharmacia) equilibrated with PBS.

4. *Absorption of conjugate to eliminate nonspecific staining.* The conjugate was absorbed with beef bone marrow to eliminate nonspecific staining of bacteria. The procedure follows: 15 grams of dried (powdered) bone marrow was used for each 100 ml. of conjugate to be absorbed. Before absorption, the bone marrow was washed with PBS and centrifuged three times for 30 minutes at 1,800 to 1,900 X gravity (*g*) or until the supernatant fluid was clear. The conjugate was then added to the packed bone marrow and mixed well. After

incubation, with frequent stirring, at 50° C. for 2 hours, the mixture was centrifuged for 30 minutes at 15,000 to 18,000 X *g*, and the conjugate was decanted.

5. *Preliminary testing.* Preliminary testing was done by using *N. gonorrhoeae* to assess specific staining, *Neisseria meningitidis* serogroup B for cross reactions, and *Enterobacter cloacae* and polymorphonuclear leukocytes for nonspecific staining. Originally, heat-treated *E. cloacae* was chosen empirically as a measure of nonspecific staining since such staining could be prevented by first absorbing the conjugate with bone marrow. This was found to be a usable assumption.

Cultures of *N. gonorrhoeae* were collected and streaked for isolation on chocolate agar plates with Difco supplement B and Thayer-Martin inhibitor (19). After the plates were incubated at 35° C. for 18 to 20 hours with CO₂, typical colonies were isolated and identified by conventional methods. (Isolates may be lyophilized and kept as stock cultures for future reference; these cultures should be lyophilized as soon as possible after isolation. A light suspension (McFarland 1) of young 18- to 20-hour *N. gonorrhoeae* cultures (fresh isolates) was prepared in distilled water, and a loopful (2 mm.) of this suspension was smeared within a 6-mm. circle on microscope slides and allowed to air dry.

E. cloacae, American-type culture collection (ATCC) No. 222, was grown on chocolate agar with supplement B and harvested in PBS. The suspension was boiled at 100° C. for 1 hour and washed three times in PBS. After the final wash, the cells were resuspended in PBS and stored at 5° C. Smears of this suspension were prepared as outlined.

Polymorphonuclear leukocytes were obtained from the urethral discharges of patients with gonococcal urethritis. Smears obtained from this material were air dried and stored at 5° C.

The conjugate was diluted in PBS in two-fold dilutions of 1:2 to 1:128. One drop (0.01 to 0.03 ml.) of each dilution of conjugate was spread over each smear (6-mm. circle) and incubated in a moist chamber at room temperature for 3 to 5 minutes. The smears were rinsed gently but thoroughly with distilled water and blotted dry. Cover slips were mounted with

glycerin, pH 9.0, prepared by adding 1 part of carbonate-bicarbonate buffer, pH 9.0, to 9 parts of glycerin.

The slides were examined under fluorescence microscope, which has an HBO-200 lamp, a BG-12 exciter filter, a darkfield condenser, and a Corning 3-72 (3387) barrier filter, using 100X objective and 10X ocular. Although this filter combination was quite satisfactory, other combinations may be equally suitable. The preliminary titer was considered to be the highest dilution showing brilliant staining (3+, 4+) of *N. gonorrhoeae*. A titer of 1:16 or higher was considered to be acceptable (table 1).

6. *Absorption with N. meningitidis serogroup B, ATCC No. 13090.* These specimens were cultured on chocolate agar with Difco supplement B, and the plates were incubated at 35° C. with CO₂ for 18 to 20 hours. The cultures were harvested in PBS with 3 percent formalin, mixed well, and incubated, with frequent stirring, for 1 hour at room temperature. The cells were then washed three times in PBS and centrifuged for 30 minutes at 1,800 to 1,900 X g after each washing. The conjugate was tested as previously outlined to determine if *N. meningitidis* would cross-react. Staining must occur if absorption is to be effective; when staining occurs, absorb the conjugate.

Ten milliliters of conjugate was added to 1 ml. of the packed *N. meningitidis* cells and incubated, with frequent stirring, for 2 hours at 50° C. The conjugate was centrifuged at 18,000 X g at 5° C. for 30 minutes, decanted, and

again tested for staining of *N. meningitidis* serogroup B, ATCC No. 13090. No staining occurred.

7. *Absorption with N. meningitidis serogroup B No. 101, Venereal Disease Research Laboratory (VDRL) stock culture.* Further absorption of the conjugate with *N. meningitidis* serogroup B No. 101 was done since the conjugate still stained organisms of this strain after it had been absorbed with *N. meningitidis* serogroup B, ATCC No. 13090. *N. meningitidis* serogroup B No. 101 was cultured on brain-heart infusion agar with 5 percent defibrinated rabbit blood and incubated as described. The cultures were harvested in nutrient broth containing 6 percent dextrose and 1 percent Difco supplement B.

The growth on 10 plates of *N. meningitidis* was harvested in 80 ml. of broth, or approximately 1 ml. of packed cells per 80 ml. of broth. The broth cultures were incubated, with frequent stirring, for 2 hours at 35° C. The cells were then washed three times in PBS and finally resuspended in PBS with 3 percent formalin and incubated at room temperature, with frequent stirring, for 1 hour. The cells were again washed three times in PBS and centrifuged at 1,800 to 1,900 X g at 5° C. for 30 minutes. Ten milliliters of conjugate was added to 1 ml. of the packed cells of *N. meningitidis* serogroup B No. 101 and incubated, for 2 hours at 50° C., with frequent stirring. The conjugate was centrifuged at 18,000 X g at 5° C., decanted, and again tested for staining of *N. meningitidis* serogroup

Table 1. Example of an antigenococcal conjugate titration before and after absorption with bone marrow

Absorption with bone marrow	Titer						
	1:2	1:4	1:8	1:16	1:32	1:64	1:128
Before:							
<i>Neisseria gonorrhoeae</i>	4+	4	4	3	2	1	Negative
<i>Neisseria meningitidis</i> B	4+	4	4	3	2	1	Negative
<i>Enterobacter cloacae</i>	1-2+						
Polymorphonuclear leukocytes	4+						
After:							
<i>Neisseria gonorrhoeae</i>	4	4	4	3	2	1	Negative
<i>Neisseria meningitidis</i> B	4	4	4	3	2	1	Negative
<i>Enterobacter cloacae</i>	Negative						
Polymorphonuclear leukocytes	Negative						

NOTE: Leaders mean that no test was made.

B No. 101. No staining occurred with *N. meningitidis* serogroup B No. 101 or with the other nine meningococcal strains tested, Nos. 102 to 110. The conjugate was filter sterilized and stored at -20°C .

8. *Titration of the absorbed conjugate.* A light suspension (McFarland 1) of young 18- to 20-hour *N. gonorrhoeae* cultures (fresh isolates) was prepared in distilled water. Smears of the suspension and twofold dilutions of the absorbed conjugate were made as described in step 5. The titer of the conjugate was considered to be the highest dilution showing brilliant staining (3+, 4+). Necessary controls for non-specific staining were included.

9. *Inhibition of Staphylococcus aureus staining.* *S. aureus*, VDRL stock strains 800, 801, 802, and 803, were cultured on chocolate agar with Difco supplement B for 18 to 20 hours at 35°C with CO_2 . A light suspension of these cultures was prepared, and a loopful was

smear on a microscope slide as described in step 5. The conjugate was diluted as outlined, added to the smear, incubated for 3 to 5 minutes at room temperature, rinsed, and examined as before. If staining occurred, the conjugate was diluted with PBS to two dilutions lower than the titer, as determined in step 8, and mixed with an equal portion of normal pooled rabbit serums that had been filter sterilized. For example, if the titer of the conjugate after absorption was 1:4 then it was diluted 1:2 with normal rabbit serums. The final dilution of 1:2 therefore was one dilution less than the titer. This procedure generally has eliminated *S. aureus* staining. Each batch of normal rabbit serums must be tested for its effectiveness in blocking *S. aureus* staining. Absorption and inhibition techniques with immune *S. aureus* antiserums also may be used.

10. *Final testing of conjugate.* *N. gonorrhoeae* isolates were cultured on Thayer-Martin

Table 2. Fluorescent antibody staining with *Neisseria gonorrhoeae* conjugate

Micro-organism	Typical results ¹	
	Conjugate absorbed with bone marrow only	Conjugate absorbed with <i>Neisseria meningitidis</i> B
Male gonococcal urethral smears (fresh) 25.....	4+	4+
<i>Neisseria gonorrhoeae</i> (fresh isolates) ² 25.....	3+, 4+	3+, 4+
<i>Neisseria meningitidis</i> A, ATCC ³ 13077.....	Occasional 4+	Negative
<i>Neisseria meningitidis</i> B, ATCC 13090.....	Occasional 4+	Negative
<i>Neisseria meningitidis</i> B, VDRL ⁴ 101.....	Occasional 4+	Negative
<i>Neisseria meningitidis</i> C, ATCC 13102.....	Negative	Negative
<i>Neisseria meningitidis</i> D, ATCC 13113.....	Negative	Negative
<i>Neisseria meningitidis</i> , VDRL 102-110.....	Occasional 4+	Negative
<i>Neisseria catarrhalis</i> , VDRL 200.....	Negative	Negative
<i>Neisseria sicca</i> , VDRL 210.....	Negative	Negative
<i>Neisseria flava</i> , VDRL 220, 221.....	Negative	Negative
<i>Neisseria perflava</i> , VDRL 230, 231.....	Negative	Negative
<i>Neisseria subflava</i> , VDRL 240.....	Negative	Negative
<i>Neisseria flavescens</i> , VDRL 250, 251.....	Negative	Negative
<i>Neisseria hemolysans</i> , VDRL 260.....	Negative	Negative
<i>Mima polymorpha</i> , VDRL 300, 301, 310, 311.....	Negative	Negative
<i>Herellea vaginocola</i> , VDRL 400, 401.....	Negative	Negative
<i>Veillonella</i> species, VDRL 500, 501, 502.....	Negative	Negative
<i>Streptococcus</i> A, VDRL 600.....	Negative	Negative
<i>Streptococcus</i> B, VDRL 601.....	Negative	Negative
<i>Streptococcus</i> C, VDRL 602.....	Negative	Negative
<i>Streptococcus</i> D, VDRL 603.....	Negative	Negative
<i>Streptococcus</i> F, VDRL 604.....	Negative	Negative
<i>Streptococcus</i> G, VDRL 605.....	Negative	Negative
<i>Staphylococcus aureus</i> , VDRL 800, 801, 802, 803.....	Negative	Negative
<i>Enterobacter cloacae</i> , ATCC 222.....	Negative	Negative

¹ Conjugate diluted 1:2 with rabbit serums.

² All cultures were 18 to 24 hours old.

³ ATCC-American type culture collection.

⁴ VDRL-Venereal Disease Research Laboratory (Center for Disease Control, Atlanta, Ga.)

NOTE: The final product may be stored, lyophilized, or frozen at -20°C .

medium. (Other bacteria, as noted in table 2, were cultured on chocolate agar with supplement B.) A light suspension of each strain of bacteria was prepared in distilled water, and smears were prepared and examined as previously outlined. After absorption with bone marrow alone or with bone marrow and meningococci, and after the addition of antistaphylococcus serum, the conjugate was tested against a variety of pathogenic and nonpathogenic strains of *Neisseria*, *Mima*, *Herellea*, *Veillonella*, *Streptococcus*, *Staphylococcus*, and *E. cloacae*. Typical results are shown in table 2.

The conjugate at the dilution needed for laboratory tests was preserved with merthiolate (1:10,000) and stored at -20°C . to -50°C . or lyophilized.

Discussion

In this experience, all fresh isolates of *N. gonorrhoeae* that were tested stained with this conjugate, although in some instances only 1 percent or less of the bacterial cells stained. Isolates conceivably might be found in which none of the cells would stain.

Nonspecific staining of polymorphonuclear leukocytes requires interpretation. For example, a urethral smear from a man with gonococcal urethritis may have polymorphonuclear leukocytes that have ingested gonococci. These will stain specifically by the FA procedure; however, if all the polymorphonuclear leukocytes stain brightly, the investigator can safely assume that nonspecific staining is occurring. This staining can be removed with beef bone marrow, as outlined in step 10. Since the final dilution of our conjugate was usually 1:2, testing of the conjugate against polymorphonuclear leukocytes and *E. cloacae* was restricted to that dilution.

The step that appears to be the most critical in the preparation of the conjugate is its absorption with meningococci. Unless small amounts of the meningococci are used for the time periods indicated, more reactivity will be removed from the conjugate than is necessary. The investigator may question the absorption of the conjugate with such a small amount of antigen. The answer is that the antigenic relationship between the meningococci and the gonococci is very close; therefore, the classic absorption method of

equal volumes of cells and serums will remove more reactivity than is necessary from a practical viewpoint (1, 20, 21).

I should also mention that meningococcal strains, other than those used in absorption, will fail to stain after absorption only to the extent that they share antigens with the strains used for absorption. Although these absorptions are necessary to render the conjugate specific for gonococci, it is obvious that if only a screening reagent is desired for detecting the presence of pathogenic *Neisseria* on a culture plate, the absorption with meningococci may be omitted.

The choice of bacterial strains for evaluating the conjugate was limited to species having a similar morphology. A distinct rod form that may react is less important. Since the hyperimmune gonococcal rabbit serums may have many antibodies that react with bacteria, other than *N. gonorrhoeae*, morphology must be considered.

Summary

Thirty fresh isolates of *Neisseria gonorrhoeae*, colonial type 1, were inoculated subcutaneously and intravenously into rabbits over a 5-week period. The globulin from the antisera produced was isolated and labeled with fluorescein isothiocyanate. This conjugate was absorbed with bone marrow and *Neisseria meningitidis* serogroup B. Rabbit antistaphylococcal serum was added to inhibit staphylococcal staining. The completed conjugate was found to stain *N. gonorrhoeae* brilliantly and specifically.

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Tearsheet Requests

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Drug Abuse Education Film Guide

A film resource guide for drug abuse education and prevention programs has been released by the National Clearinghouse for Drug Abuse Information, National Institute of Mental Health, Health Services and Mental Health Administration.

The guide, "Selected Drug Abuse Education Films," lists 13 films available on topics ranging from the nature, treatment, and control of drug addiction to drug abuse in relation to broader social issues such as poverty, inner city life, and the alienation of youth. Some of the films are intended for the general public while others are appropriate for high school students in drug education programs. Many titles are available on free loan.

This preliminary guide will be enlarged to a definitive film catalog which will be available from the Clearinghouse in fall 1970. Single courtesy copies are available. Address requests to Film Guide, National Clearinghouse for Drug Abuse Information, 5454 Wisconsin Ave., WT-240, Chevy Chase, Md. 20015.