

ABSTRACT

THE APPLICATION OF FLUORESCENT ANTIBODY TECHNIQUES AND THE USE OF A BIPHASIC ENRICHMENT MEDIUM FOR THE IDENTIFICATION OF NEISSERIA GONORRHOEAE

Waldemar Meyer

The use of Fluorescent antibody (FA)-techniques as a routine diagnostic procedure for the identification of Neisseria gonorrhoeae was evaluated.

It was found that fluorescent antigonococcal rabbit globulin absorbed with an equal volume of formalin killed cells of Neisseria sicca for one hour in a 50°C waterbath, and then diluted to its staining titer with human serum could be used to stain N. gonorrhoeae specifically with the exception of Neisseria meningitidis.

Six strains of N. gonorrhoeae which were exposed to concentrations of penicillin ranging from 0.075 units to 40 units per ml. could be stained and identified with FA even where cells were swollen and distorted. Photographs of stained and swollen cells were taken.

The reliability of finding and identifying gonococcal cells in actual clinical specimens with the FA-technique was examined by staining 70 urethral smears from a selected group of males showing definite symptoms of a gonococcal infection. A total of 69 positive identifications could be made by the FA-technique as compared to 67 by direct microscopy.

Using a biphasic media as an enrichment media resulted in 26 positive identifications out of 37 urethral swabs from males and 15 positive identifications out of 38 urethral swabs from females. N. gonorrhoeae was identified in 24 males and 10 females by conventional methods. Out of a total of 43 (100%) cases of N. gonorrhoeae detected by both methods 95.3% were identified by the biphasic - FA - method and 79.3% by the conventional technique.

The liquid media yielded 0.7 to 1.0 gm of N. gonorrhoeae (dry weight) in simple shake cultures.

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NEISSERIA GONORRHOEAE

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I. INTRODUCTION

1. Definition of the Problem

Most Laboratories today consider the presence of Gram-negative diplococci in a direct smear from the urethra, vagina, or cervix, presumptive evidence of gonococcal infection. In the case of males showing definite clinical symptoms of an acute gonococcal urethritis, the physician may not request any further tests, particularly if the organisms are intracellular.

However, in patients with chronic infections, and in females who often show no signs of a gonococcal urethritis this presumptive evidence must be confirmed by culturing the organism, and subsequent fermentation tests. Failure to find any Gram-negative diplococci in the direct smear does not rule out the possibility of a gonococcal infection, and a culture may often result in a positive diagnosis. Thus to confirm a gonococcal infection and to obtain a higher rate of detection swabs of each patient should be streaked on chocolate agar plates or preferably chocolate agar plates containing ristocetin and polymyxin B as suggested by Thayer and Martin (28). The Thayer Martin medium permits selective growth of Neisseria gonorrhoeae by inhibiting or reducing the growth of other species of neisseria, and bacterial contaminants. The selection of a particular colony by its morphology for further fermentation tests is difficult as different strains may have different colonial appearances (18), therefore the ability of N. gonorrhoeae to produce oxidase is used to recognize possible gonococcal colonies. The agar plate is flooded with oxidase reagent,

which turns colonies of the neisseria group pink within a few minutes.

Members of the enterobacteriaceae family which cannot be differentiated from members of the neisseria group by fermentation tests do not react with oxidase reagent and can be eliminated at this point. The presence of oxidase positive colonies with a Gram stain of the culture showing Gram negative diplococci is taken as presumptive cultural evidence of N. gonorrhoeae which must be confirmed by fermentation tests. Organisms from oxidase positive colonies are streaked onto dextrose, maltose, and sucrose slants, and if only dextrose is fermented the organism is conclusively identified as N. gonorrhoeae.

It thus requires at least two days to confirm a gonococcal infection by these procedures. Because of the time required to complete a diagnostic report by conventional methods a technique would be desirable which would permit the identification of N. gonorrhoeae with equal or greater accuracy in a shorter time and possibly at reduced cost.

The introduction of fluorescent antibodies (FA) into the diagnostic laboratory seems to provide such a technique. With fluorescent antibodies an organism can be identified within an hour in a mixed flora containing a ratio of contaminating organisms to specific cells as high as 10^7 to 1 without great difficulty (29). This sensitivity and the savings in time and labor compared to conventional methods prompted many investigations for the use of FA as a diagnostic tool. In 1959 Deacon et al.(9) developed a FA technique for

N. gonorrhoeae.

It is the purpose of this thesis to evaluate and to describe the development of FA techniques for the identification of these organisms. Also described is the development of a biphasic medium to increase the number of positive identifications in symptomless females.

Also examined was the effect of penicillin on fluorescent staining of N. gonorrhoeae.

II. REVIEW OF LITERATURE

1. Principle and Development of FA - techniques

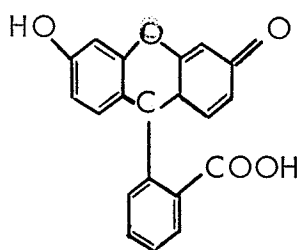
The use of FA to localize and identify bacteria depends, as in the case of agglutination reactions, on the specific immunochemical reaction between antibodies and antigens. But FA methods have the advantage over agglutination tests in that bacterial cells can be localized and identified within one hour. Bacteria are exposed to their homologous antibodies which are labeled with a fluorescent dye, and the resulting antibody-antigen complex is made visible by viewing it under a fluorescent microscope.

Heidelberger (16) in 1933 was the first to introduce a colored label into the antibody-antigen complex. He coupled the red chromophore 2-naphthol - 3,6 - disulphonic acid through a bidiazo linkage to egg-albumin which could then be determined by spectrophotometric methods. In 1934 Marrack (20) extended Heidelberger's experiment by labeling antityphoid and anticholera antibodies with a red dye and showed that homologous organisms were colored specifically by these labeled antibodies.

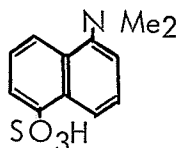
In 1941 Coons (3) confirmed the findings of Marrack by specifically staining and agglutinating pneumococci types 2 and 3, but found that the intensity of this colored label was barely visible against the background color of biological tissues. To increase the intensity of the color antibodies were labeled with a fluorescent dye.

Fluorescent molecules, called fluorochromes, are substances which are

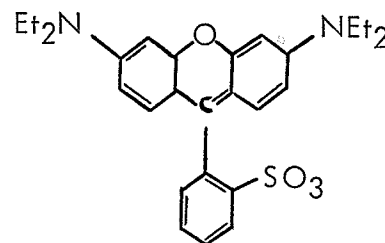
able to absorb light energy in the ultraviolet to blue range by shifting electrons to higher orbitals and are able to return those electrons to their ground state during exposure to light energy with an emission of visible light of longer wave length. Several such fluorochromes have been used as labels. Dyes most commonly used as labels are fluorescein, rhodamine, and 1 - dimethylamino-naphthalene-5 - sulfonic acid, (DANS).



Fluorescein



1-Dimethylamino-naphthalene-5-sulfonic acid

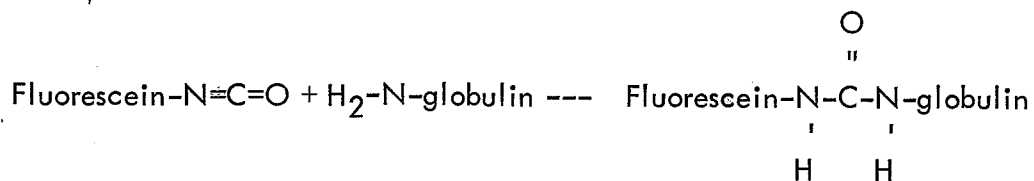


Lissamine Rhodamine

A particular colored label may be selected to give maximum contrast to background tissue. Polyvalent antisera can be prepared by labeling different antibodies with labels of different color. Using a polyvalent antiserum makes it possible for example, to label antistreptococcal A globulin with green fluorescein and antipneumococcal type 3 globulin with a red rhodamine and identify both organisms in one smear.

Because of its distinct apple green color under ultra violet light fluorescein imparts good contrast against most biological tissues and has become the most widely used label since its introduction by Coons, Creech, Jones, and Berliner in 1942 (4). They coupled fluorescein-iso-cyanate to anti-

pneumococcal type 3 rabbit serum and showed that type 3 pneumococci were stained by this conjugate, and that type 2 pneumococci remained unstained on exposure to the same serum.



Agglutination titers of the antiserum with or without the label remained unchanged at 1:800 with 1 to 2 molecules of fluorescein attached per molecule of protein.

But because fluorescein-iso-cyanate decomposes readily in water (5) anhydrous reagents and a dry environment are required for the conjugation process to reduce the loss of fluorescence to a minimum.

A more stable compound was found in fluoresceinisothiocyanate ($\text{R} = \text{N} = \text{C} = \text{S}$) which could easily be conjugated to protein (27). By labeling seven different antisera with fluoresceinisocyanate and fluorescein-iso-thiocyanate, Marshall, Eveland, and Smith (21) showed that antibodies conjugated with the latter gave higher fluorescent intensities. For example, antiserum against Pasteurella tularensis conjugated with fluoresceinisothio-cyanate diluted 1:80 had the same intensity as a 1:5 dilution of the same antiserum labeled with fluoresceinisocyanate. The advantage of conserving antiserum is obvious. But the main advantage of fluoresceinisothiocyanate is that it can easily be conjugated to proteins without the use of organic solvents which may further denature the globulin (21).

2. FA - Techniques

Four different FA-techniques have been developed; direct, indirect, inhibition, and complement staining (2).

The direct method is the original method developed by Coons (4). Tissue sections containing the antigen or bacterial smears are fixed to a microscope slide using a fixative which preserves the particular antigenic structure best. The fixed bacterial smear is flooded with FA and incubated for periods from 15 to 60 minutes (22), and excess (unreacted) material is washed off with a buffered solution.

A more sensitive and versatile technique is that of indirect staining developed by Weller and Coons (30). Indirect staining is carried out in two steps. The fixed antigen is first treated with unlabeled specific antibody followed by a labeled antibody against the first antibody, which acts also as an antigen. For example, the antigen may be treated in the first step with rabbit antibody, the complex formed is made visible in the second reaction by treating it with labeled goat-anti-rabbit globulin.

Even though this technique requires more time and labor it has several advantages over the direct method.

Deacon (8) used this technique to detect treponemal antibodies in patients examined for syphilis. Here smears are made with known Treponema pallidum antigens which are then flooded with the patients serum followed by labeled antihuman goat globulin. A fluorescent reaction indicates the

presence of treponemal antibodies in the patients serum. An other advantage is that one kind of labeled antibody can be used to stain a great variety of antigens.

Complement staining is also done in two steps. Unlabeled complement and unlabeled antibody are allowed to react with antigen. The resulting product is made visible with labeled anticomplement globulin. Goldwasser and Shepard report complement staining to be more sensitive than indirect staining (14).

The inhibition, or blocking, technique is used to identify antibodies in an unknown serum. A known antigen is treated with an unknown serum and subsequently with its homologous labeled antibodies. If the serum contains antibodies against the fixed antigen the antibodies will combine with it and block the union of that antigen with the labeled antibodies. An unstained product indicates the presence of specific antibodies in the test serum.

3. Optical Equipment for Fluorescent Microscopy

The stained bacterial smear or tissue section, prepared by any of the above techniques is mounted by placing a drop of buffered glycerol saline solution on the slide and placing a cover slip on top. As fluorescein, like many other dyes, changes the intensity of its color with a change in pH, the mounting fluid is buffered at a pH of 7.2. White and Kellogg (31) report that adjustment of the mounting fluid to pH 8.4 helped to maintain an intense fluorescent color.

The mounted specimen is now placed under a fluorescent microscope for examination.

The fluorescent microscope is a standard microscope equipped with a darkfield condenser, and an ultra-violet-blue light source. A Mercury arc lamp with an emission spectrum from 280 to 600 $m\mu$ is suitable to stimulate fluorescence in most fluorochromes (23). Radiating heat is greatly reduced by a filter in the lamp housing. A primary transmission filter which may be selected to transmit light of a desired wave length is placed between lamp and specimen. Under the stimulus of this selected wave length the label emits fluorescent radiation. A secondary, or barrier filter, placed between the specimen and the eye of the microscopist provides a black background and protects the eyes from harmful radiation by removing radiation up to a wave length of 430 $m\mu$. Because the emission of fluorescent light is above this wave length the label remains visible.

A schematic diagram constructed from a drawing by Nairn (24) showing the components of the fluorescent microscope and the principles of fluorescent staining is shown in Fig. 1.

4. Antigenic Analysis of *Neisseria gonorrhoeae*

The simplicity of the FA-technique is due to the fact that the only requirement for identifying an organism is the reaction between antibody and antigen. Thus apart from morphology the only factor to identify an organism with FA is its antigenic structure, whereas identification by conventional

FLUORESCENCE MICROSCOPE

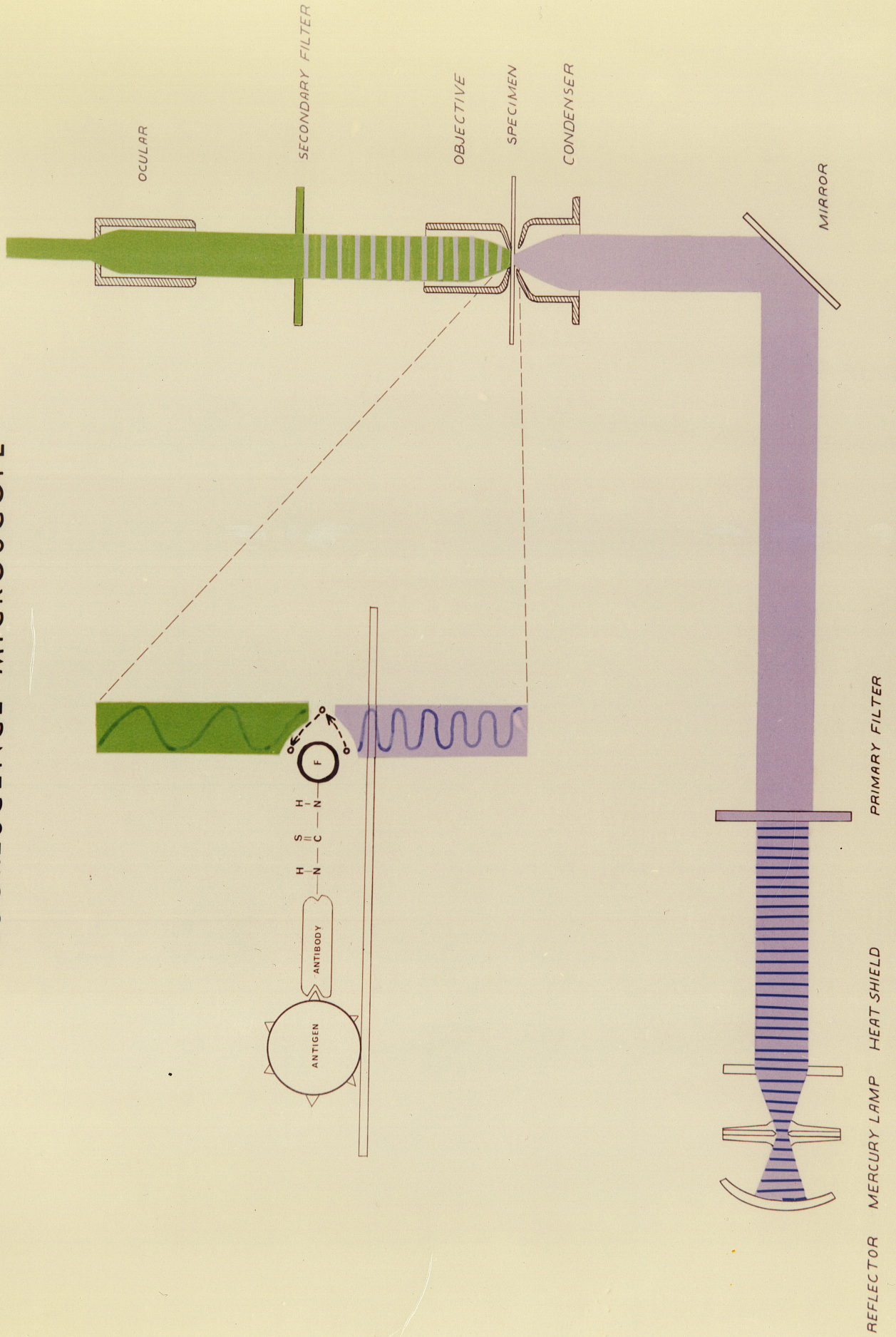


Fig. 1 Schematic Diagram showing components of the fluorescence microscope and principles of fluorescent antibody staining. (modified from Nairn)

methods relies on several factors e.g. Gram stain, morphology, colonial appearance and biochemical reactions.

It is the dependence on one single character which may question the reliability of the FA technique. For failure of the reaction would make it impossible to give any information on the organism at all or a false negative report would be made. A cross reaction with other organisms would result in a false positive report.

Thus before FA can be applied to the identification of a particular organism an extensive study of the organism's antigenic structure must be made.

In an antigenic analysis of N. gonorrhoeae Wilson (32) demonstrated in 1954 by agglutination and agglutinin absorption tests 8 antigens in "smooth" strains. "Smooth" (capsulated) strains were only isolated from primary cultures and were frequently found to be inagglutinable by homologous antisera. Inagglutinability was lost on subculturing, and Deacon et al. (9) suggested "that the 'factor' responsible for inagglutinable gonococcus cultures could be the most important diagnostic element of the N. gonorrhoeae cell". By slide agglutination tests they showed that this factor was preserved by 3% formalin but destroyed by a temperature of 100°C. Antibodies produced in rabbits against formalin killed cells were conjugated with fluorescein isothiocyanate. The conjugate was then applied to N. gonorrhoeae, N. meningitidis, and other organisms of the neisseria group. Several strains of N. meningitidis stained as intensely as N. gonorrhoeae, and N. catarrhalis showed a weak

cross reaction. The high degree of cross - reaction between N. meningitidis and N. gonorrhoeae was found to be due to the sharing of common antigens (33). Absorption of the antiserum with N. meningitidis greatly reduced or eliminated the nonspecific staining.

The newly recognized capsular antigen became the basis for the identification of N. gonorrhoeae and today antiserum is prepared commercially against strains having this antigen (11).

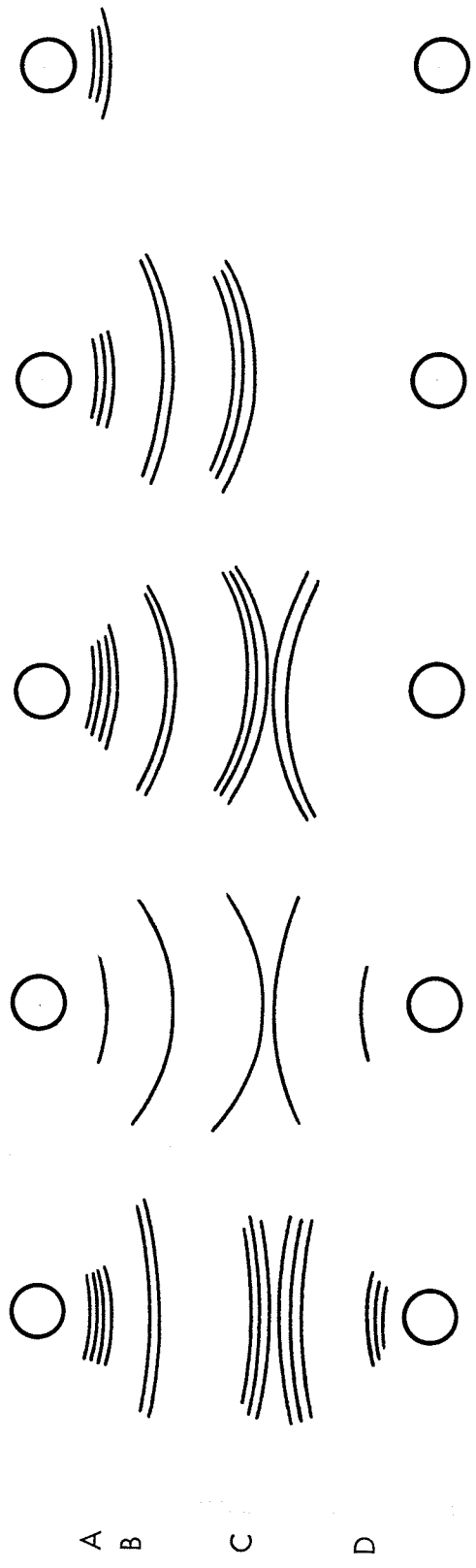
But as cells which have lost this capsule on repeated subculturing can be stained with FA produced against capsulated cells, other antigens must also be involved in the reaction. Danielson (6) in 1965 showed in an extensive study of N. gonorrhoeae the involvement of heat stable as well as heat labile antigens in FA staining. He also studied the antigenic relationship between N. gonorrhoeae and other strains of neisseria by gel diffusion and absorption tests. Various species of the neisseria group were sonicated and the disintegrated cells were used for gel diffusion tests against a reference serum produced against formalin killed whole gonococci. The serum produced 15 lines against N. gonorrhoeae which were divided into 4 groups, A, B, C, and D. Heat killed gonococci showed a total of 5 lines with 1 or 2 lines in each group. Organisms of other species did not produce any lines in group D, but all shared antigens in group A. Antigens in groups B and C are probably intracellular components as absorption of the reference serum with whole cells did not remove these lines but removed lines in group A. Antigenic factors in

group D and partially those from group A take part in the FA-staining reaction. As A antigens are common to all species, and are in an extracellular position, this group may be the cause of cross reactions. To determine the degree of cross reactions Danielson in 1965 stained the test strains with the fluorescein labeled reference serum at different dilutions. The staining titer, that is the degree to which the serum could be diluted without loss of staining intensity, was 1:256 for N. gonorrhoeae, 1:64 for heat-killed N. gonorrhoeae and N. meningitidis, and less than 1:2 for N. sicca, N. flava, N. catarrhalis, and N. flavescens. A diagram (fig. 2) showing the antigenic relationship of the neisseria group and the staining titers of the reference serum for each organism was compiled and constructed from data obtained by Danielson (6).

5. FA applied to Diagnosis of N. gonorrhoeae

One of the advantages of the FA-technique is that it can identify a single nonviable organism, which makes it possible to diagnose pathogens in a direct smear without further cultural procedures. Deacon (9) showed that N. gonorrhoeae could easily be identified in direct urethral smears from males using FA. However direct vaginal, cervical, or urethral smears from fifty named female contacts gave only 26% positive results compared to 58% positive cultures. To increase the sensitivity of detection Deacon (10) developed the Delayed Fluorescent Antibody (DFA) technique. Swabs taken from the same sites were planted immediately on chocolate agar slants by rubbing and rotating them over the agar surface and leaving them in contact

Antiserum produced against formalin killed gonococci



Antigens:	N. gonorrhoeae	N. gonorrhoeae (heat killed)	N. meningitidis	N. sicca N. flava	N. catarrhalis
Staining Titer:*	1:256	1:64	1:64	less than 1:2	less than 1:2

Figure 2. Precipitin lines of sonicated strains of Neisseria formed against an antigenococcal reference and staining titers of fluorescein labeled reference serum.

* Limit to which fluorescein labeled reference serum could be diluted and still retain max. (4+) staining.

with the media. The slants were placed in candle jars at 35C for 16 to 20 hours, and then used to make heavy smears. This enrichment resulted in 58% positive identification in the above study group.

The usefulness of the DFA-technique in a venereal disease control program was studied by examining 213 female jail inmates (15). Swabs were again taken from urethra, vagina, and cervix, and only from inmates who after a speculum examination showed no symptoms of a gonococcal infection. 20.3% of the women in this group were found to harbor N. gonorrhoeae.

Even though the DFA-technique shortens the cultural procedures by eliminating the fermentation tests, a reliable examination of direct smears is still desirable, and a different approach was made to increase the number of positive findings in direct smears by using a bacteriological loop to collect specimens from the cervix. The smears were treated as before, but to reduce staining of leucocytes and mucus which stains non specifically or react with uncombined fluorescent dye, thus obscuring the recognition of specific organisms, the smears were counterstained. Using flazo-orange as a counterstain Peacock and Thayer (26) obtained 54% positive results in a study group of 156 female contacts, compared to 46% without counterstain and 56% by cultural techniques. In an other study using Evans blue as a counterstain White and Kellogg (31) equaled or exceeded the number of positive cultures. They divided 155 specimens into three groups and cultured each group by a different technique; 58 specimens in group I were inoculated directly onto GC-medium base, 37

specimens from group II onto GC-media base containing 10 μ g/ml of ristocetin and 25 units/ml polymyxin, and 60 specimens in group III were collected in GC-media without agar but containing the antibiotics used in group II. The collected specimens were stored in a candle jar at room temperature until they were delivered to the laboratory within 4 hours. Here the 60 specimens collected in the liquid were centrifuged and subcultured onto GC-media plates.

N. gonorrhoeae was identified in 48% of the cultures in group I, and 62% in each of the cultures from groups II and III by fermentation tests and FA. Duplicate direct smears stained with FA and Evans blue as a counterstain were positive in 59% of the cases in groups I and II and in 63% of the smears from group III.

A modification of the direct technique is the "rapid direct" method developed by Kellogg and Deacon (17), which can be performed almost as fast as a Gram stain. Danielson (7) compared a modified "rapid direct" method, where a smear is treated with preheated (37°C) conjugate for 2 minutes at 56°C and then rinsed for a few seconds; to direct microscopy, direct FA-, DFA-, and cultural techniques, and identified 74 cases of gonorrhoeae by rapid direct, 55 by direct microscopy, 74 by direct FA, 69 by DFA, and 59 by cultural techniques out of 100 patients.

6. Observations on Literature

Evidence in the literature supports the belief that FA may play an important role in the identification of *N. gonorrhoeae* once the problems of

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cross reaction have been solved. Deacon (9) effectively eliminated cross reactions by absorbing the antiserum with cross reacting organisms, and Danielson (6) showed that nonspecific staining may be reduced or eliminated by diluting the antiserum to its staining titer.

Even though FA may be applied to identifying gonococci in direct smears, eliminating cultural procedures, Deacon (10) considers a direct smear unreliable and suggests that a negative direct smear be confirmed by the DFA-technique. However White and Kellogg (31), and Danielson (7) found the direct smear equal or superior to either DFA- or cultural techniques. It must be borne in mind that the method by which specimens are collected is important in contributing to the success of any laboratory test, and the use of a bacteriological loop to make direct smears may have resulted in the greater number of positive findings in the reports of Peacock and Thayer (26), and White and Kellogg (31). Considering the fact that a direct smear may contain only a small number of organisms which may be missed during a microscopic examination and that by placing the swab on an enrichment media the number of organisms could be significantly increased as to lead to a positive identification, a DFA technique along with a direct smear would give the maximum number of positive identifications.

In a schematic diagram comparing the FA-technique to the conventional one (Fig. 3) it can be seen that direct smears stained with FA can be diagnosed conclusively within one hour if fluorescent diplococci are found, and only

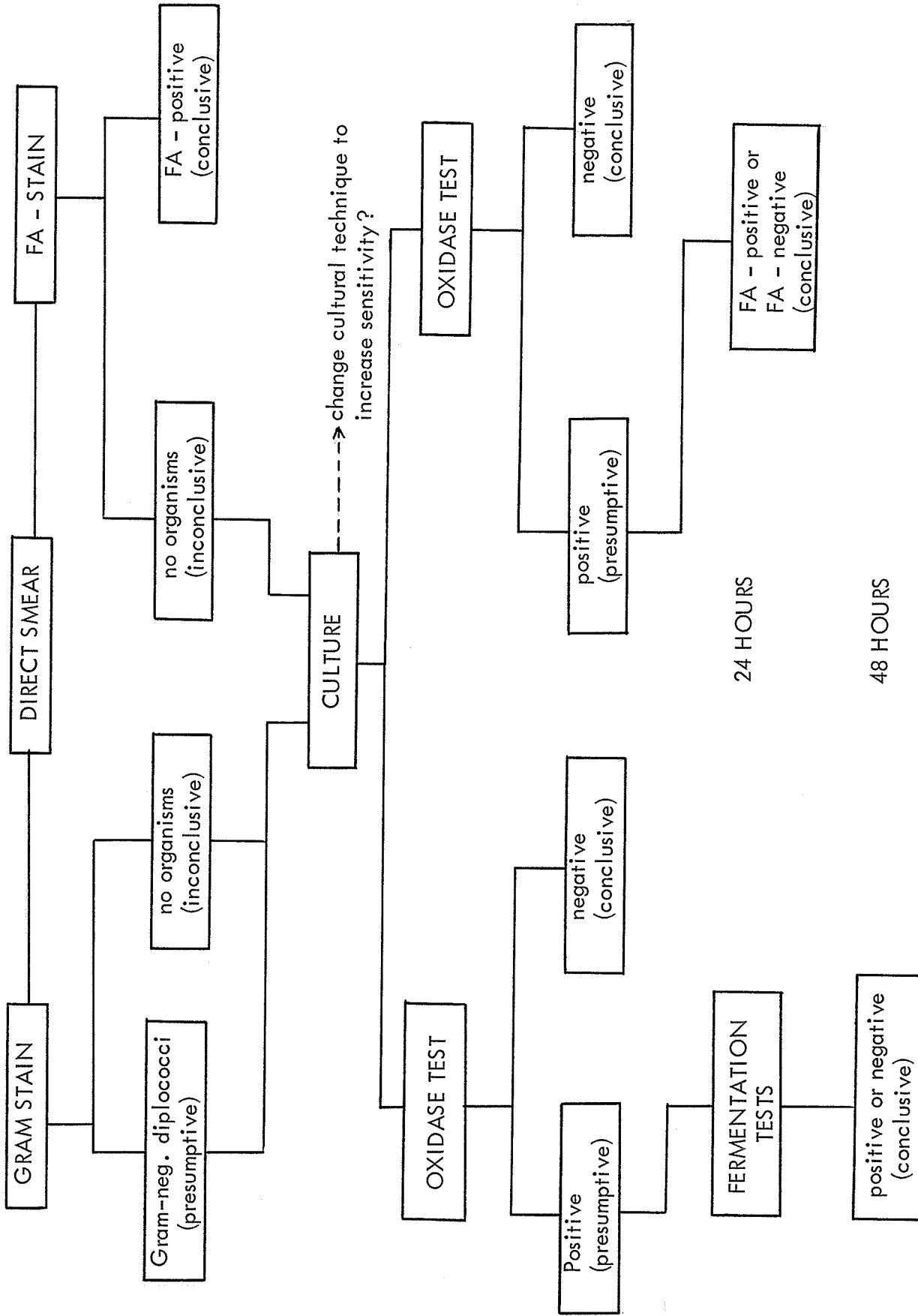


Figure 3. Comparison of specimens processed for the identification of *N. gonorrhoeae* by conventional (left) and FA - techniques (right)

smears which show no fluorescent organisms have to be confirmed by culture, whereas all conventionally Gram stained smears, whether they show Gram-negative diplococci or not have to be cultured for a conclusive report. It can also be seen that it takes at least 48 hours to confirm a gonococcal infection by conventional methods, and that by replacing the fermentation tests with FA-Staining to confirm oxidase positive colonies from culture plates an equivalent diagnosis can be made at least 24 hours earlier.

Because the organisms need not be isolated in pure culture for their identification by FA, a different cultural technique may result in a greater number of positive identifications possibly in a shorter time.

III. MATERIALS, METHODS AND RESULTS

1. Materials

Microscope: A Leitz fluorescence unit with a "Laborlux" microscope was used. The light source consisted of an Osram HBO 200 mercury vapor lamp with a Schott 1.5 mm BG 12 blue excitor filter and an K 490 barrier filter unless stated otherwise. All examinations were made with a 2 mm. oil immersion objective.

Reagents: Fluorescein conjugated rabbit anti-gonococcus globulin was commercially prepared and purchased from Difco Laboratories. For use the globulin was rehydrated with distilled water and absorbed with 0.15 gm per ml of Difco 2390 FA bone marrow in a 50°C waterbath for 2 hours. The bone marrow was then removed by centrifugation at 15000 rpm for 30 minutes.

Organisms: Cultures of N. gonorrhoeae and other neisseria were isolated and identified by fermentation tests at the diagnostic laboratory of the Winnipeg General Hospital. One strain of Mima polymorpha 9957, and one strain of Neisseria meningitidis (serogroup A) 13077-p were obtained from the American Type Culture Collection.

2. Experimental Methods

The FA-technique is based on two principles:

- (a) The ability of antibodies to react with their homologous antigen.
- (b) The phenomenon of fluorescence; which may be defined

as the ability of some substances to emit visible light when stimulated by light of shorter wave length in the ultra-violet to the blue range.

The first objective was to examine variables which may influence or effect one or both of the above principles involved so as to derive at a maximal staining with the minimum of time and labor.

i. Incubation periods and dilution of antiserum

A colony of 24 hours culture of N. gonorrhoeae was suspended in phosphate buffered saline, and a loopful of the suspension was spread on microscope slides within 9 to 10 mm circles drawn with a marking pencil. A total of six slides each containing 5 circles were prepared, air dried for about 30 minutes, heat fixed, and exposed to various dilutions of FA for different time intervals at 37°C in a moist chamber to prevent evaporation of antiserum. The smears were removed at pre-determined time intervals, rinsed and flooded with phosphate buffered saline of pH 7.2 for 10 minutes, blotted dry with paper towels, and mounted with a mounting fluid buffered at the same pH. The degree of staining intensity varied with dilution of FA and incubation period (Table I). Subsequent organisms were exposed to fluorescent antiserum diluted with human serum 1:4 for periods ranging from 45 to 60 minutes.

ii. pH of buffer and mounting fluid

Many dyes react to a change in pH by changing their color or the intensity of their color. The effect of hydrogen ion concentration on fluores-

TABLE I

THE EFFECT OF INCUBATION TIME AND DILUTION OF ANTI-GONOCOCCUS GLOBULIN UPON FLUORESCENCE INTENSITY OF N. GONORRHOEAE

Incubation Periods	Dilution of anti-gonococcus globulin				
	Undiluted	1:2	1:4	1:8	1:10
5 minutes	3+ *	1+	1+	0	0
15 minutes	3+	3+	1+	0	0
30 minutes	4+	3+	2+	1+	0
45 minutes	4+	4+	4+	1+	0
60 minutes	4+	4+	4+	2+	1+
120 minutes	4+	4+	4+	3+	2+

* Degree of fluorescent intensities.

0 = not fluorescing

1+ = trace of fluorescence

2+ = weak fluorescence

3+ = bright fluorescence

4+ = brilliant fluorescence

cent intensity of fluorescein was examined by using phosphate buffered saline solutions over a range from pH 5.7 to 8.1 to rinse the stained smear and then mounting the smear in fluid adjusted to the same pH.

A .25M solution of primary sodium phosphate (solution A), a .25M solution of secondary sodium phosphate (solution B), and a 17% saline solution was prepared as described by Estelle and Shuey (12). Solutions A and B were mixed in varying proportions and added to a constant volume of 5 ml saline solution. The final volume of each mixture was brought to 100 ml with distilled water. The pH of each solution was measured, and mounting fluids of corresponding pH were prepared by mixing 4.5 ml of glycerol with 0.5 ml of each corresponding buffer and by adding either a few drops of solution A or B to bring the mounting fluid to the appropriate pH.

A suspension of N. gonorrhoeae was used to prepare and stain ten smears. Each smear was washed in a different buffer and mounted in the corresponding mounting fluid. The fluorescent intensity of each smear was examined under three different combinations of filters and recorded in Table II. Subsequent stained smears were washed for 8 to 10 minutes with phosphate saline solution and mounted in mounting fluid buffered at pH 8.0.

iii. Mode of fixation

Air dried smears of N. gonorrhoeae were fixed with 3% formalin saline for 10 minutes, 95% ethanol or acetone for 2 minutes, and by heat. Slightly higher fluorescent intensities could be observed on organisms fixed

TABLE II
EFFECT OF pH ON FLUORESCENT INTENSITIES

Composition of Buffers **			Combination of filters		
Solution A	Solution B	pH	BG 12 K 490	BG 12 K 460	BG 3 (Primary) K 490 (Secondary)
NaH ₂ PO ₄ ·H ₂ O (ml.)	Na ₂ HPO ₄ ·H ₂ O (ml.)				
3.6	0.4	5.7	3+ *	3+	3+
3.0	1.0	6.2	3+	3+	3+
2.5	1.5	6.5	3+	3+	3+
2.0	2.0	6.7	3+	3+	3+
1.0	3.0	7.1	3+	3+	3+
Commercial Fluid		7.2	3+	3+	3+
0.5	3.5	7.5	4+	3+	3+
0.3	3.7	7.7	4+	4+	4+
0.2	3.8	7.8	4+	4+	4+
0.1	3.9	8.1	4+	4+	4+

* Degree of fluorescent intensities
 0 = not fluorescing
 1+ = trace of fluorescence
 2+ = weak fluorescence
 3+ = bright fluorescence
 4+ = brilliant fluorescence

** 5 ml of 17% NaCl solution was added to each Buffer and the total volume was then brought to 100 ml with distilled water.

with 95% ethanol, but the difference was considered insignificant and subsequent smears were fixed by heat because of ease of handling.

iv. Control of Cross-reactions.

Antibodies against a particular organism may react with other organisms having a similar antigenic structure. As any reaction of other organisms with FA could result in a false positive diagnosis, potentially cross reacting organisms were exposed to undiluted anti-gonococcal globulin for 1 hour, and their staining intensities recorded in Table III.

Staining was found to be specific for N. gonorrhoeae except for the two strains of meningococci which stained as intensely as gonococci, 1 strain of Staphylococcus aureus, and 1 strain of N. sicca each staining with 1+ intensity. By diluting the antiserum 1:4 with normal human serum the cross reaction with Staph. aureus could be eliminated and reduced with N. sicca. The cross reaction with N. sicca could be removed completely by absorbing the antiserum with an equal volume of formalin killed cells of the cross-reacting strain in a 50°C waterbath for 1 hour, but the cross reaction with meningococci could not be removed by absorption without also reducing the staining titer for gonococci significantly.

v. Preparation of enrichment media for Neisseria gonorrhoeae.

Smears made from swabs which were incubated on chocolate agar slants with or without ristocetin and polymyxin B were very thick due to other contaminating organisms, making it difficult to find gonococci. To facilitate

TABLE III

FLUORESCENT INTENSITIES OF ORGANISMS EXPOSED TO UNDILUTED
ANTI-GONOCOCCAL GLOBULIN FOR 1 HOUR

Organism	No. of Strains tested	Degree of fluorescence intensity *				
		4+	3+	2+	1+	0
<i>N. gonorrhoeae</i>	4	4 **	-	-	-	-
<i>Staph. aureus</i>	3	-	-	-	1	2
<i>N. catarrhalis</i>	5	-	-	-	-	5
<i>N. flavescens</i>	14	-	-	-	-	14
<i>N. sicca</i>	6	-	-	-	1	5
<i>N. meningitidis</i>	2	2	-	-	-	-
<i>Neisseria (other)</i>	4	-	-	-	-	4
<i>Mima polymorpha</i>	1	-	-	-	-	1

* 0 = not fluorescing
 1+ = trace of fluorescence
 2+ = weak fluorescence
 3+ = bright fluorescence
 4+ = brilliant fluorescence

** Number of strains attaining a particular degree of fluorescent intensity.

the finding of organisms and to conserve antiserum it is desirable to confine organisms to a small restricted area on the microscope slide. Because organisms which are grown in a liquid medium may be concentrated by centrifugation, attempts were made to grow gonococci in a liquid or in a biphasic (solid media layered with a liquid) medium.

The media used was essentially that described by Gerhardt and Hedén (13) but was modified to support growth from a small inoculum, as from urethral swabs, and consisted of 1.5% proteose peptone No. 3, 0.2% dextrose (autoclaved separately), 0.5% NaCl, 0.3% $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$, and 2% gelatin. One ml. of citrated sheep blood was added to 10 ml. aliquots of sterilized broth in 8.0 x 2.2 cm. universal bottles. This mixture was placed in a 80° C waterbath for 10 minutes, poured into centrifuge tubes and centrifuged at 3000 rpm. for 30 minutes. The supernatant was poured into flasks and stored as "GC-Broth" at room temperature until needed. The broth was divided into 4 lots; one lot was enriched with 1.0% supplement A (Difco), the second with 1.0% "Isovitalex" (BBL), the third lot contained 1.0% "Isovitalex", 10 µg/ml. ristocetin and 25 units/ml. polymyxin B, and the fourth lot consisted of plain "GC-Broth".

3. Experimental Results

i. The effect of oxidase reagent on fluorescent staining

All strains of N. gonorrhoeae exposed to oxidase reagent, a 1.0% aqueous solution of p-amino-dimethylalanine monohydrochloride, retained their stainability with FA.

ii. The effect of antibiotics on fluorescent staining

(a) Action of Penicillin

As penicillin acts on the cell wall of N. gonorrhoeae an experiment was set up to determine if organisms exposed to penicillin could still react with their homologous antibodies and be identified by the FA-technique.

Six strains of N. gonorrhoeae were exposed to penicillin in concentrations ranging from 40 units per ml. to 0.075 units per ml. The plates were streaked with suspensions of each strain and incubated in 5% CO₂ (candle jars) for 24 hours at 37°C. Impression smears were made of all plates showing no visible growth by cutting blocks of agar of about 1 x 2 cm and inverting them on microscope slides for about 20 minutes. Colonies from plates showing growth, including colonies from control plates without penicillin, were suspended in approximately 1 ml of saline, and a loopful of the suspensions was spread inside 8 to 10 mm circles on microscope slides. All smears were air dried for at least 30 minutes, heat fixed, and stained with FA. Three strains of N. sicca, and one strain of Mima polymorpha were included in this series to examine the possibility of penicillin altering the antigenic structure or exposing cross reacting antigens in these organisms.

All smears were carefully examined for the number of organisms present, their morphology, and their staining intensity.

At concentrations between 40 and 5 units of penicillin only isolated cells ranging from 0.5 to 4 μ in diameter could be found, and many of them

were highly distorted. There was an increase in number of cells with decreasing concentrations of penicillin. Most cells could be found on plates containing a minimal inhibition concentration (MIC) of penicillin.

Small cells were stained evenly and as intensely as the control organisms. Inflated cells were stained less but contained highly fluorescent spots and minute granules, giving the cells a spotted appearance, (Fig. 4, 5, 6, 7, 8, 9) but specific staining was retained by all strains. No organisms were found in impression smears taken from plates inoculated with N. sicca or Mima polymorpha.

(b) Action of Ristocetin and Polymyxin B

10 μ g/ml of ristocetin and 25 units of polymyxin B are widely used to selectively grow N. gonorrhoeae in primary cultures. To examine if ristocetin and polymyxin B had an adverse effect on fluorescent staining 3 strains of N. gonorrhoeae were grown on 5 μ g/ml ristocetin with 12.5 units/ml polymyxin B, 10 μ g/ml ristocetin with 25 units/ml polymyxin B, and 20 μ g/ml ristocetin with 50 units/ml polymyxin B. There was no difference in stainability compared to organisms grown on plates without antibiotics.

iii. Application of FA to detect N. gonorrhoeae in clinical specimens

After determining that gonococci could be stained specifically with FA, with the exception of meningococci, and that gonococcal cells which had been exposed to antibiotics could be stained and identified, the reliability of the FA-technique to detect and identify N. gonorrhoeae in actual

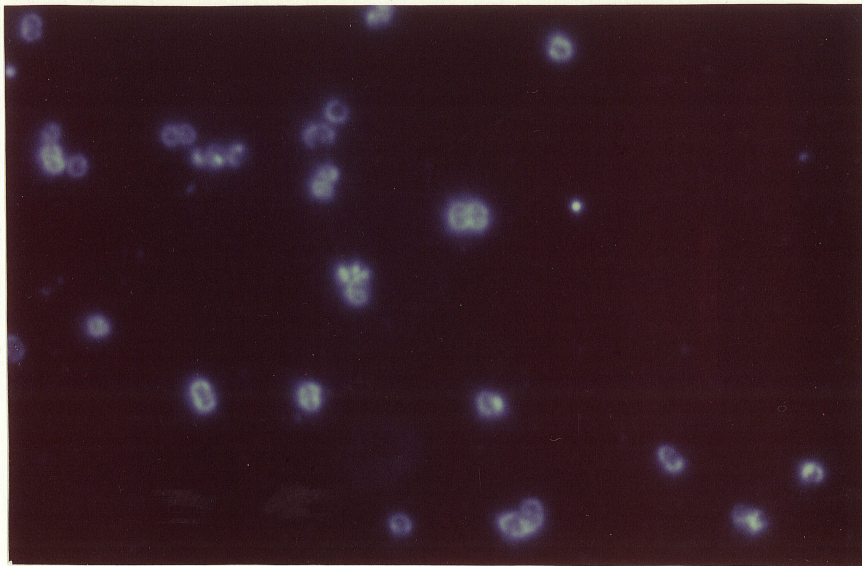


Fig. 4. Unstained cells of N. gonorrhoeae. The blue color is autofluorescence of the organism. (x 1800)

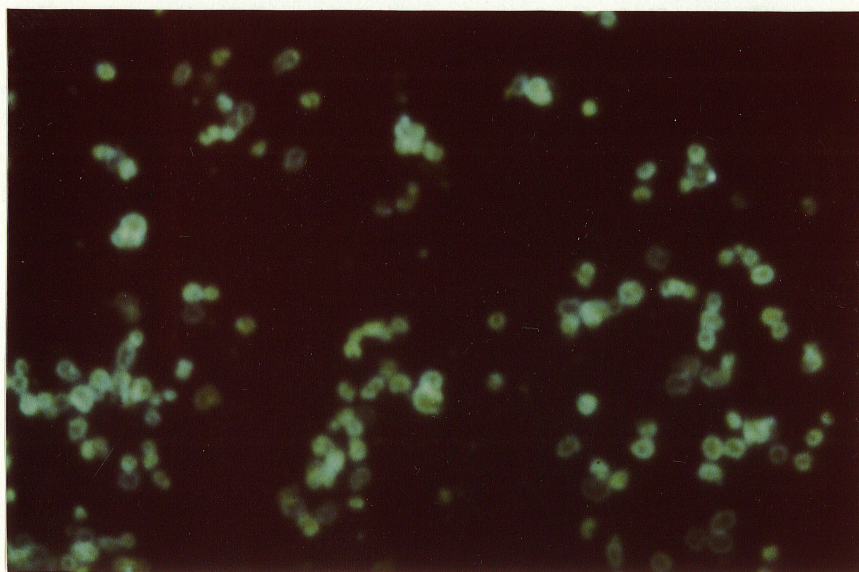


Fig. 5. N. gonorrhoeae stained with fluorescent antibodies. (x 1800)

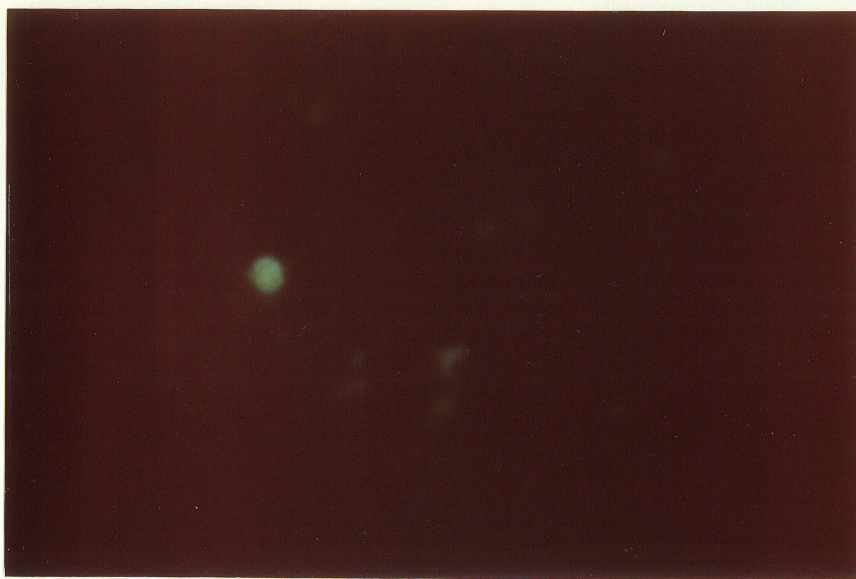


Fig. 6. Gonococcal cell exposed to 20 units/ml of penicillin for 24 hours and stained with FA. Note cellular debris of lysed cells in the background. (x 1800)

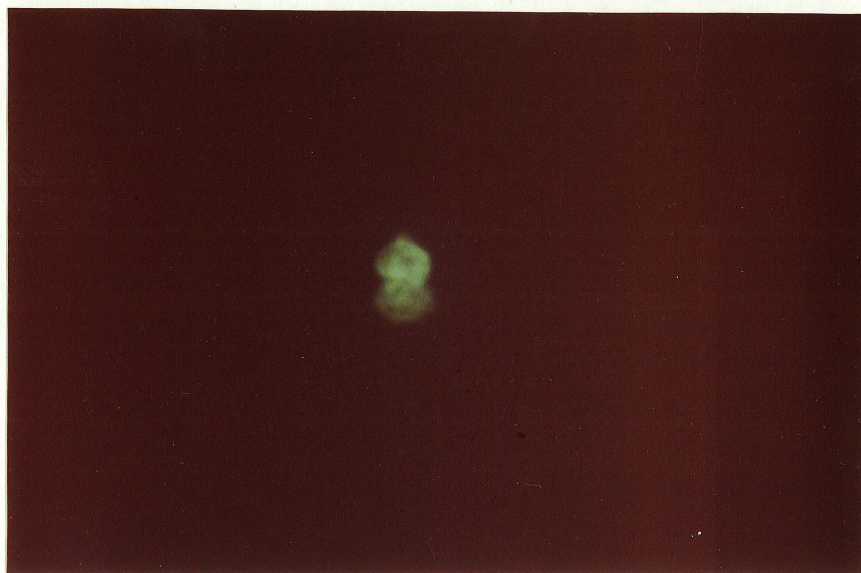


Fig. 7. FA-stained diplococci of *N. gonorrhoeae* slightly swollen due to exposure to 10 units/ml. of penicillin for 24 hours. (x 1800)

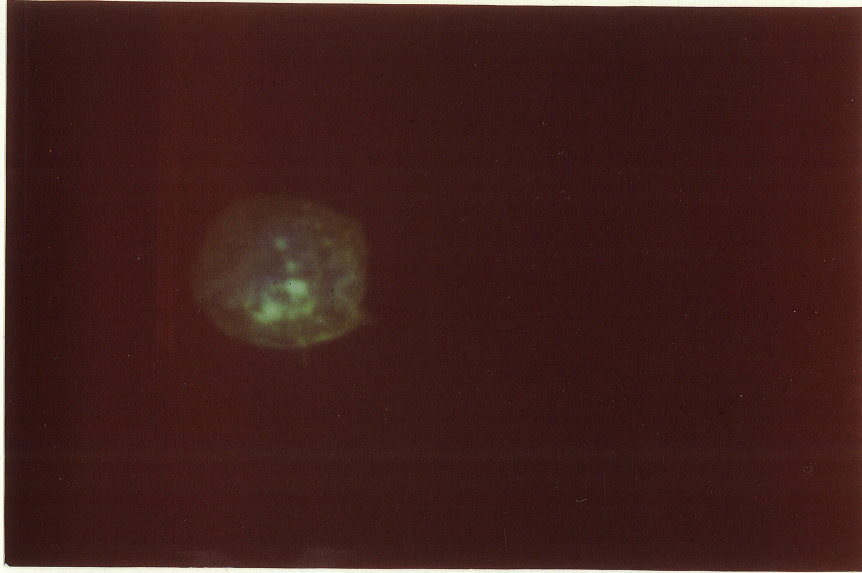


Fig. 8. A single cell of *N. gonorrhoeae* swollen due to exposure to 0.315 units/ml of penicillin for 24 hours, and stained with FA. (x 1800)

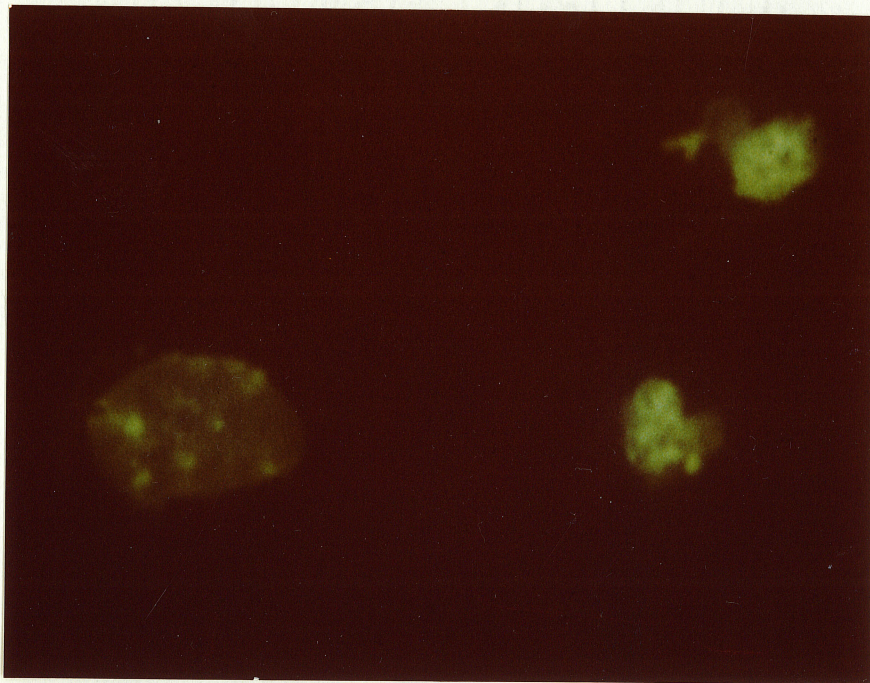


Fig. 9. Gonococcal cells swollen and distorted due to exposure to 0.315 units/ml of penicillin for 24 hours, and stained with FA. (x 2400)

clinical specimens was established.

A direct smear and a swab were taken from the urethra in a selected group of 70 males showing definite symptoms of a gonococcal infection. The direct smears were heat fixed, and stained with fluorescent antigonococcal serum, which had been absorbed with N. sicca and diluted 1:4, for periods from 45 to 60 minutes.

The swabs were received in Stuart's Transport media about 18 hours after collection, at that time they were placed on chocolate agar slants containing ristocetin and polymyxin B and incubated for 16 to 18 hours in candle jars at 37°C to enrich the specimens with organisms, as suggested by Deacon (10). The swabs were then used to make heavy smears. The "delayed" smears were stained in the same manner as the direct smears and examined for N. gonorrhoeae.

Most direct smears were diagnosed within 1 to 2 minutes with only 1 smear out of 69 requiring 4 minutes, the maximum time that we thought should be taken for efficient routine diagnostic work. A total of 70 smears were examined over a period of 2 months. Table IV shows the results, which were compared to results of a duplicate series examined by direct microscopy at the St. Boniface Hospital. Both methods agreed on 67 positive and 1 negative smear. Two more positive identifications could be made by the FA-technique than by direct microscopy.

However smears made by the delayed fluorescent antibody technique

TABLE IV
 IDENTIFICATION OF GONOCOCCI IN DIRECT
 URETHRAL SMEARS FROM MALES

Number of paired direct smears	Results of 2 techniques				Combined Number of Positives	
	FA-techniques	+	-	+		-
	Direct Microscopy	+	-	-	+	
70		67	1	2	0	69

required 5 to 6 minutes of scanning to make a positive identification, and 10 smears of 40 smears - only 40 swabs out of the total of 70 were received within 18 hours and were considered to contain viable gonococci - could not be confirmed at all by this method.

As these results are in disagreement with the findings of Deacon (10) who reported that enrichment of swabs gave more than twice as many positive results as the direct smear, and since the diagnosis of N. gonorrhoeae in asymptomatic females may often depend on increasing the number of organisms in a suitable enrichment media, the next sections of this chapter will describe the growth of N. gonorrhoeae in liquid and biphasic media, and the use of a biphasic medium for the DFA-technique.

iv. Growth of Neisseria gonorrhoeae in the enrichment media

A volume of 12.5 ml of each media was pipetted into 125 ml Erlenmeyer flasks, and a duplicate series was layered on 50 ml chocolate agar with or without the two supplements or antibiotics. Each flask received an inoculum of 22880 cells per ml (strain 14079) and was incubated in 5% CO₂ (candle jar) at 37°C, on a rotary shaker at 120 revolutions per minute.

Viable count made after 16 hours and 40 hours (TABLE V) show that the biphasic system supports denser populations of gonococci than the corresponding liquid media. Highest densities were obtained with media containing 1% "Isovitalex".

Even though the addition of ristocetin and polymyxin had a retarding

TABLE V
GROWTH OF NEISSERIA GONORRHOEAE (STRAIN 14079)
IN LIQUID AND BIPHASIC MEDIA

	Media *	Viable counts per ml.	
		16 hours $\times 10^7$	40 hours $\times 10^9$
Liquid	GC - Broth (plain)	6.34	2.21
	GC - Broth & 1% suppl A	0.67	1.01
	GC - Broth & 1% "Isovitalex"	10.0	**
	GC - Broth & 1% "Isovitalex" & ristocetin and polymyxin B	6.11	2.35
	Chocolate agar (plain)	8.56	contaminated
Biphasic	Chocolate agar & 1% suppl. A	5.87	contaminated
	Chocolate agar & 1% "Isovitalex"	10.7	contaminated
	Chocolate agar & 1% "Isovitalex" & ristocetin and polymyxin B	7.13	178.00

* The solid layer of the biphasic system consisted of 25 ml of chocolate agar containing the same concentrations of 1% suppl. A, 1% "Isovitalex", and 10 μ g/ml ristocetin and 25 units/ml of polymyxin B as the liquid media. The volume of the liquid was 12.5 ml.

** Growth of gonococci was too dense to obtain counts at a dilution of 10^6 .

effect on the rate of growth of N. gonorrhoeae further studies were made with media enriched with 1% "Isovitalex" but containing these antibiotics to inhibit the overgrowth of contaminants from clinical specimens. Liquid and biphasic media were prepared as before and inoculated with 4 strains of N. gonorrhoeae. To examine if the total surface area of media exposed to CO₂ had an effect on the growth rate 80 x 26 mm universal bottles containing an equal volume of media were included in this series. Viable counts were made again after 16 and 40 hours (TABLE VI). The media in the flasks forming a thin layer of about 4 mm yielded 10 to 160 times as many cells as the 30 mm column of the same media in universal bottles. Highest densities were obtained with the biphasic system.

To examine if the liquid broth could be used to grow large quantities of gonococci, for possible future immunological and biochemical work, 24 hour colonies of strains M8 and 14079 were inoculated into 100 ml GC-broth enriched with 1% "Isovitalex" in 500 ml Erlenmeyer flasks. The flasks were placed in a candle jar and shaken on a rotary shaker at 120 revolutions per minute at 37°C. Dry weight determinations after 24 hours resulted in yields from 0.7 to 1.005 gm per liter.

vi. The use of a biphasic enrichment media on clinical specimens

Duplicate urethral swabs were taken from a random group of 37 males and 38 females who had come to the Venereal Disease Clinic of the St. Boniface Hospital for an examination. The swabs were kept in Stuart's transport media

TABLE VI

GROWTH OF 4 STRAINS OF NEISSERIA GONORRHOEAE
IN 3 DIFFERENT SYSTEMS

Strain	Inoc./ml	Viable counts per ml.					
		Universal Bottle		125 ml Flask		Biphasic	
		16 hr $\times 10^9$	40 hr $\times 10^{12}$	16 hr $\times 10^9$	40 hr $\times 10^{12}$	16 hr $\times 10^9$	40 hr $\times 10^{12}$
14 079	8 080	0.0880	0.0058	0.835	1.08	01.5	(3) **
14 587	1 250	0.284	0.0100	3.12	2.46	**	07.3
M 8	2 492	0.1070	0.0137	16.20	229.00	1280.0	(4) **
M 9	6 408	0.2130	0.0154	(1) **	(2) **	263.0	98.6

** Growth of gonococci was too dense to obtain counts at dilutions of

(1) 10^6 (2) 10^8 (3) 10^9 (4) 10^{10}

for no longer than 8 hours. One swab was used to streak Thayer-Martin plates, and one was placed in about 1.5 ml of "GC-broth" which had been layered on chocolate agar slants in universal bottles. The chocolate agar and the GC-broth contained 1% "Isovitalex", 10 µg/ml ristocetin, and 25 units/ml polymyxin B. The slants were incubated with the swabs in a candle jar at 37°C in a slanted position to cover the whole agar surface with a thin layer of liquid for 14 to 16 hours, after which the liquid phase was poured into 10 x 70 mm test tubes. The cells were concentrated by centrifugation for 15 minutes and resuspended in a few drops of residual supernate after discarding the top layer. A loopful of the suspension was spread evenly into 8 to 10 mm circles made with a grease pencil, and the smears were stained as described earlier. The Thayer-Martin plates streaked with duplicate swabs were reincubated for an other 24 hours, because most plates showed no potential gonococcal colonies after 14 hours, and were then flooded with oxidase reagent. Colonies turning pink were used to make smears which were stained with FA. The latter series was considered the conventional method, the results of which were compared to the biphasic enrichment series. In the case of 37 paired swabs from males, 23 were positive for N. gonorrhoeae by both methods, 10 negative by both methods, 3 were identified by biphasic only, and 1 by conventional technique only.

Of the 38 paired swabs from females, 9 were positive for N. gonorrhoeae by both methods, 22 were negative by both methods, 6 were identified by the biphasic technique only, and 1 by the conventional technique only (TABLE VII).

TABLE VII
 COMPARISON OF CONVENTIONAL CULTURE AND DELAYED FLUORESCENT
 ANTIBODY TECHNIQUE USING A BIPHASIC SYSTEM

Number of Paired Swabs	Biphasic Conventional	Results of 2 techniques				Combined Number of Positives
		+	-	+	-	
37 Males		23	10	3	1	27
38 Females		9	22	6	1	16
75 Total		32	32	9	2	43
Percentage of cases of N. gonorrhoeae		74.4%		20.9%	4.7%	100%

IV. DISCUSSION AND SUMMARY

1. Discussion

Most investigations of the nutrient requirements of N. gonorrhoeae were conducted to grow these pathogens on solid media to obtain isolates required for fermentation tests. Because FA-techniques permit the identification of specific organisms in mixed cultures it may now be possible to simplify or shorten cultural procedures by growing N. gonorrhoeae on liquid or biphasic media.

The biphasic medium was selected as an enrichment medium because the system gave higher yields of gonococci than the corresponding liquid medium. Incubating the slants on an incline resulted in optimal growing conditions by forming a large contact area between the liquid phase and the solid layer, and between liquid and CO₂ environment. Organisms could diffuse from the swab into the liquid where they multiplied to numbers easily detectable under the fluorescent microscope when concentrated by centrifugation. The biphasic media made it possible to shorten incubation periods to 16 hours as compared to 40 hours by conventional plates where gonococcal colonies required from 18 to 40 hours to attain sizes visible to the eye.

An important finding is that the biphasic media gave 7 more positive results than the conventional culture out of a total of 43 cases of N. gonorrhoeae. This is significant in view of the fact that cultural procedures developed in 1964 by Thayer and Martin (28) have equaled or surpassed

Deacon's DFA-technique in the number of positive identifications (19).

The sensitivity of FA-staining was demonstrated by exposing gonococci to various concentrations of penicillin before staining them with FA. It was found that gonococcal cells which were swollen and distorted attained brilliant fluorescent intensities. Even though this sensitivity supports the use of FA-techniques for the identification of N. gonorrhoeae, it may question the value of FA for follow up examinations. Lucas et al. (19) report that 27 out of 772 previously untreated patients gave positive DFA reactions 2 to 4 days after treatment. Subsequent examinations could confirm only 4 cases by culture and 6 by fluorescent antibodies.

Apart from the use as an enrichment media for a DFA-technique, a liquid media which meets the growth requirements of various strains of N. gonorrhoeae may lead to a procedure for identifying or typing of N. gonorrhoeae by serological or biochemical means other than fermentation tests which are considered unreliable (25). Only 2 strains out of a total of 43 failed to grow from the urethral swabs placed on the biphasic enrichment media. Duplicate swabs of the 2 biphasic negative specimens streaked on Thayer-Martin plates showed only 1 and 5 colonies of N. gonorrhoeae indicating the possibility of no viable gonococcal cells being inoculated. On the other hand 9 strains of N. gonorrhoeae which failed to grow on Thayer-Martin plates grew in the biphasic enrichment media.

The same liquid media capable of growing large quantities of gonococci

under reproducible conditions could be used for the production of antigens for immunological research. Yields of 0.7 to 1.0 gm dry weight per liter obtained by simple 100 ml shake cultures compares favorably with yields of 0.1 to 0.3 grams per liter obtained by Brookes and Hedén (31) in 100 ml control cultures using a different liquid medium.

2. Summary

The use of FA-techniques as a routine diagnostic procedure for the identification of N. gonorrhoeae was evaluated.

It was found that fluorescent antigenococcal rabbit globulin (Difco) absorbed with an equal volume of formalin killed cells of N. sicca for one hour in a 50°C waterbath, and then diluted to its staining titer with human serum could be used to stain N. gonorrhoeae specifically with the exception of N. meningitidis.

The smears were air dried for at least 20 minutes, heat fixed, and flooded with fluorescent antiserum for periods from 45 to 60 minutes. Increasing the incubation period from 30 minutes with undiluted antiserum to 45 minutes with antiserum diluted 1:4 to obtain maximum (4+) staining can be justified by more efficient use of the serum, and reducing the possibility of cross reactions. Cross reactions with Staph. aureus could be eliminated at this dilution.

Fluorescent intensities could be improved by adjusting the buffered solution, and the mounting fluid to pH 8.0.

Six strains of N. gonorrhoeae which were exposed to concentrations of penicillin ranging from 0.075 units to 40 units per ml. could be stained and identified with FA even where cells were swollen and distorted.

The reliability of finding and identifying gonococcal cells in actual clinical specimens with the FA-technique was examined by staining 70 urethral smears from a selected group of males showing definite symptoms of a gonococcal infection. A total of 69 positive identifications could be made by the FA-technique as compared to 67 by direct microscopy.

Growth studies of N. gonorrhoeae showed that a biphasic media may be used to increase the number of gonococci in a specimen. Using a biphasic media as an enrichment media resulted in 26 positive identifications out of 37 urethral swabs from males and 15 positive identifications out of 38 urethral swabs from females. N. gonorrhoeae was identified in 24 males and 10 females by conventional methods. Out of a total of 43 (100%) cases of N. gonorrhoeae detected by both methods 95.3% were identified by the biphasic - FA - method and 79.3% by the conventional technique.

The liquid media yielded 0.7 to 1.0 gm of N. gonorrhoeae (dry weight) in simple shake cultures.

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