Spring 1979

The Use Of The Indirect Fluorescent Antibody Test As A Serological Test For Culturally-Confirmed Gonorrhea

David Minehan
Carroll College

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THE USE OF THE INDIRECT FLUORESCENT
ANTIBODY TEST AS A SEROLOGICAL
TEST FOR CULTURALLY-CONFIRMED
GONORRHEA

Submitted in Partial Fulfillment of the
Requirements for Graduation with Honors
to the Department of Biology at Carroll
College, Helena, Montana

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March 30, 1979
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March 30, 1979
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ABSTRACT

The use of the indirect fluorescent antibody test as a serological test for gonorrhea was investigated. The best results were shown using strain F62 of Neisseria gonorrhoeae as antigen at a serum titer of 1:16 in which, out of a total sample of seventeen, 71% correct predictions occurred. In this case the greatest error was seen in predicting false negative results. Strain N9, out of a sample size of eighteen, predicted only 50% of the cases correct at a titer of 1:16. The serum dilution of 1:16 appeared to be the most diagnostic titer to use.
A. History

Galen, in 130 A.D., first introduced the term gonorrhea (gono + Gr. rhein to flow) which meant "flow of seed" (38). It was not, however, until the thirteenth century that physicians started using the term to describe the medical conditions characterized by urethral discharge which were transmitted venereally. Gonorrhea was often confused with syphilis, another venereally transmitted disease, because often the two were acquired simultaneously. The great physician, John Hunter, in 1767, mistakenly ascribed syphilitic symptoms to gonorrhea after acquiring both syphilis and gonorrhea during an autoinoculation experiment and thinking he had only gonorrhea. The two diseases were not effectively differentiated until the middle of the nineteenth century. In 1885, Neisser isolated an organism which proved to be the causative agent of gonorrhea. The organism was identified as a bacterium and was given the genus name Neisseria, in honor of Neisser, and the specific name, gonorrhoeae, because of the disease it caused. Man serves as the only known natural host for this bacterium, although experimental animals can be artificially infected (2).

B. Morphology

Neisseria gonorrhoeae, the gonococcus (GC), occurs as diplococci. Gonococci have no detectable capsule in vitro, are Gram negative, are not motile and do not produce spores. They vary in size from about 6μm to 1.5μm in diameter.

Structurally, the gonococcus is similar to other Gram negative bacteria. The cell envelope is composed of three major components: the cytoplasmic membrane, the rigid peptidoglycan layer and the outer
membrane which contains lipopolysaccharide phospholipid and proteins which are capable of eliciting an immunologic response.

The gonococcus forms a nonpigmented, grayish white colony after 20-24 hours incubation (some may take 40-48 hours). Colonies are raised, opaque, moist, convex, finely granular and have a shiny appearance.

C. Physiology

Optimum growth of N. gonorrhoeae occurs under aerobic conditions, with an increased carbon dioxide tension and on a moist medium surface. The Modified Thayer-Martin (MTM) media (33,34), allows for recognition of N. gonorrhoeae from materials which may be contaminated with other bacterial flora (31). This medium consists of a chocolate agar modified by the addition of vancomycin to inhibit Gram positive bacteria, colistimethate to inhibit Gram negative enteric bacteria, nystatin for the inhibition of yeasts and trimethoprim lactate to inhibit Enterobacteriaceae. Most nonpathogenic Neisseria fail to grow on this medium and only rarely are gonococci inhibited.

Gonococci utilize glucose and the resulting acid produced comes primarily from the oxidative phosphorylation pathway and not by fermentation. Facultative anaerobic respiration can occur under certain conditions. All Neisseria routinely produce catalase and cytochrome oxidase. Free iron (from heme) and nicotine adenine dinucleotide (NAD) are necessary for growth. Optimum growth temperature is 36-37 C and optimum pH is 7.2 to 7.6.

To differentiate N. gonorrhoeae from other species of Neisseria, carbohydrate tests are used. N. gonorrhoeae produces acid (no gas) from glucose, but does not ferment maltose, sucrose or lactose. These and other characteristics are shown in Table 1. Also, immunological
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<th>N. sicca</th>
<th>N. subflava</th>
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Table 1. Characteristics Differentiating the Species of Genus Neisseria (38).
studies can be performed and the different surface antigens identified. The species specific antigens may be used to identify particular species of Neisseria.

D. Determinants of Pathogenicity

The antigenic makeup of N. gonorrhoeae is complicated and not fully understood. So far, only two major classes of antigens have been detected: the pili protein antigen and a polysaccharide antigen found in the lipopolysaccharide of the cell wall. The different surface antigens are associated with different colony types.

Four major types of colonies, designated T1, T2, T3, and T4, help distinguish pathogenic forms from nonpathogenic forms (20). The existence of a fifth type has also been postulated (18). Types 1 and 2 are considered virulent. They are small, dense and predominate in primary isolates. Nonselective subculture allows for rapid emergence of types 3 and 4, which are considered non-virulent. These types are larger and more granular than types 1 and 2 (20).

There are three main characteristics of the organisms found in type 1 and 2 colonies which account for their virulence. The first characteristic is the pili. The pili are composed of helical aggregations of tubelike structures, approximately 70Å in diameter and 2μm in length having a molecular weight of about 23,000. The pili are serologically similar. Colony types 1 and 2 are composed of cells which have an enhanced ability to adhere to host cells and each other. This ability may allow cells to attach to the human urethra in spite of the flow of urine.

The second characteristic is that organisms from the virulent colony types are less likely to be ingested by macrophages as com-

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1See materials and methods for further differentiation of the colony types.
pared to the non-virulent strains. This would enhance their pathogenicity.

Thirdly, in association with the pili are small spherical structures which may contribute to the damage of the host in a way that is not yet clear. These spherical structures are more prevalent in the virulent strains than they are in the non-virulent strains. Other appendages found in colony types 1 and 2 have also been investigated to determine whether or not they are relevant to the virulence of gonococci (17).

E. Epidemiology

Gonorrhea is the most prevalent of the classical venereal diseases and is now the number one reportable communicable disease in the United States (1) (Fig. 1). The last two decades have shown a worldwide increase of epidemic proportions in the incidence of gonorrhea (Fig. 2).

Warfare and changes in social mores have contributed to increases and decreases of gonorrhea. The problem of non-recognition and the reluctance of physicians to report gonorrhea have been long-standing obstacles in the fight for control of gonorrhea and the other venereal diseases (30). Soon after World War II, the reported cases of gonorrhea per 100,000 population peaked at slightly less than 300. It was not until the late sixties that this figure was exceeded due to changes in social mores that occurred during the sixties. Reported cases of gonorrhea in the United States in 1976 reached an all time high of over one million cases (6).

On a yearly basis, incidence of gonorrhea increases through the summer months (Fig. 3). Persons 20-24 years old show the highest incidence of gonorrhea followed closely by the 15 to 19 and 25 to 30 age groups. The incidence of gonorrhea is highest among non-white individuals, persons of low socioeconomic status and urban dwellers.
Fig. 1. Communicable Diseases - Number of Reported Cases (United States, Calendar Year 1975). (1).
Fig. 2. Gonorrhea: Reported Cases. (United States: Fiscal Years 1940-1976). Courtesy of Venereal Disease Control Division, Bureau of State Services, Center for Disease Control, U.S. Public Health Service.
Fig. 3. United States Reported Cases of Gonorrhea by Four-week Intervals, Jan. 1972 to Aug. 1978. Courtesy of Center for Disease Control.
F. Pathogenesis and Etiology

Post mortem examinations of people who have died of related complications during the acute phase of gonorrhea show that the columnar epithelium of the urethra and the periurethral ducts and glands are the primary sites where infection begins (38). Cervical, conjunctival and rectal mucosa may also serve as sites for primary infections. The bacteria anchor themselves by their pili and within one hour after initial contact the infection is established. The bacteria penetrate through the intercellular spaces, invade the subepithelial connective tissue by the third day and cause an inflammatory response as polymorphonucleate leukocytes (PMNs) infiltrate the area. There is a release of gonococcal endotoxin which mixes with the serum to produce a yellow color. This yellow color and mass of cells produce the yellow, purulent (pus) discharge characteristic of gonorrhea. The dense mass of cells can also block ducts and glands resulting in the formation of cysts and emboli. The emboli may rarely detach and spread the infection over the whole body. The lymphatic vessels are the primary pathway for the disease to spread while the blood vessels play a lesser role.

Close examination reveals that the initial contact of the organism with the host cells is by way of microvilli. Gonococcal pili then sweep over the entire cell, penetrate the epithelium and disrupt the collagen connective tissue underneath. Epithelial cells are characteristically destroyed which further facilitates more rapid penetration by the gonococci.

G. Clinical Manifestations

The clinical manifestations of gonorrhea are diverse and easily confused with other disorders (1). The most characteristic symptom in the
male is a burning sensation during urination and, as mentioned before, a yellow, purulent discharge. Also, the subject usually has a low fever and high white blood count. Complications include urethral strictures, epididymitis, prostatitis, septicemia, peritonitis and meningitis. It was believed that eleven percent of the infected males remain asymptomatic, although still able to transmit the disease. Handsfield (15), in a recent study, found that as many as forty percent of the male contacts with women with pelvic inflammatory disease were bacteriologically positive for gonorrhea, albeit asymptomatic, and reinfecting their partners. Other investigators have also documented the existence of asymptomatic gonorrhea (25,26). Approximately a twenty two percent chance exists that an unprotected male will acquire gonorrhea from an infected female. Transmission is almost exclusively by sexual contact. A condom reduces the chance of transmission considerably. In general, the groups with the highest risk of being exposed to gonorrhea have the highest amount of asymptomatic gonorrhea (32).

Eighty percent of the women infected by the gonococcus remain asymptomatic or have minor symptoms not leading to medical treatment. In symptomatic females, signs of the disease include burning or frequency of urination, vaginal discharge, fever and abdominal pains (22). Gonococcal infection can cause pelvic inflammatory disease (PID) in fifteen to seventeen percent of women infected with the gonococcus. Salpingitis, the localization of the inflammation to the fallopian tubes, results in scars within the fallopian tubes which can (1) block the passage of the ova down the tube - in essence sterility and (2) block the normal flow of fluid through the tubes, thus providing a site for other bacterial infections to occur. Patients seriously ill with complications of gono-
coccal infection show endocarditis, meningitis, Fitz-Hugh-Curtis syndrome, proctitis, pharyngitis, skin lesions and even arthritis (1, 30).

A significant number of cases of gonorrhea each year occur in infants and children. Nonvenereal inoculation can occur, most notably in ophthalmia neonatarum, in which the disease is contracted during the infant's passage through the birth canal. The eyes are most susceptible and the best procedure is prophylactic treatment of the eyes of the newborn with one percent silver nitrate, as is required by law (38).

H. Laboratory Diagnosis

Microscopic examination of samples from purulent materials which have been treated with the Gram stain show the Gram negative diplococci engulfed within PMNs. The single best test for diagnosing gonorrhea in women is the cervical culture (1). Both the cervical and rectal cultures are used for test of cure. Samples from the cervix of a female must be examined cautiously because of the existence of morphologically similar saprophytic organisms (31).

Cultures are grown on the MTM medium when there is a risk of contamination with other bacterial flora. On forty percent of the cultures which eventually become positive, growth will not occur until 40-48 hours. An oxidase test is then performed. Ninety five percent of the colonies judged presumptively positive by the oxidase test and the finding of Gram negative diplococci are later confirmed to be N. gonorrhoeae by carbohydrate fermentation tests.

At present there are no serological tests which are useful for detection of antigonococcal antibodies in the diagnosis of gonorrhea,

\(^2\)A perihepatitis characterized by fever, upper quadrant pain, tenderness and spasm of the abdominal wall and occasional friction rub over the liver (30).
although there have been a number of serological studies on patients with gonorrhea. Detectable antibody appears approximately seven to ten days after initial infection and may be detectable for weeks or months after treatment (28). The diagnosis of acute gonococcal arthritis depends on the detection of gonococcal antibodies in serum (16). Humoral antibody of the IgG class is detectable by the time the patient has clinically apparent disease. IgM antibodies are usually not detectable in the early stages of the disease. IgA can be detected early in the urethral secretions (8,9). Cell-mediated immunity (CMI), as evidenced by lymphocyte blastogenesis, can occur in patients with uncomplicated gonococcal urethritis.

I. Treatment

There are four drug regimens recommended by the Center for Disease Control (CDC) for treatment of uncomplicated gonococcal infections in men and women (4). These are (1) aqueous procaine penicillin G (APPG), 4.8 million units injected intramuscularly at two sites, with one gram of probenecid by mouth, (2) tetracycline hydrochloride, 0.5g by mouth four times a day for five days (given at least one hour before or two hours after meals), (3) ampicillin, 3.5 g, or amoxicillin, 3.0 g either with one gram probenecid by mouth or (4) spectinomycin hydrochloride, 2.0 g, in one intramuscular injection for patients who cannot tolerate tetracycline. For patients allergic to penicillin or probenecid, tetracycline should be used (if tolerated).

Penicillinase producing Neisseria gonorrhoeae (PPNG) are resistant to penicillin and the drug of choice is spectinomycin hydrochloride (2 grams, IM). The first case reported of PPNG in the United States was in March 1976. Since then 402 cases have been reported. PPNG strains
resistant to spectinomycin may be treated with cefoxitin (2.0 g, IM with 1.0 g probenicid).

J. Statement of Thesis

A serological method for the diagnosis of gonorrhea would be useful because (1) it is difficult to obtain an adequate endocervical culture from women, (2) endocervical cultures from women with asymptomatic gonorrhea fail to provide diagnosis in twenty percent of the cases (38), (3) nearly eighty percent of women and ten percent of men with gonorrhea are asymptomatic and (4) it would permit epidemiological study of gonorrhea. Blood samples could then be tested for syphilis and gonorrhea at the same time.

The work reported in this paper is concerned with the determination of the ability of the IFA technique to detect gonococcal infection. The accuracy of this serological test can then be compared to cultural analysis.
MATERIALS AND METHODS

A. Cultural Samples

The gonorrhea and blood samples used in this study were obtained from clinical specimens sent to the Microbiology Laboratory at the Montana Department of Health and Environmental Sciences, Helena, Montana, for laboratory diagnosis. Isolates, obtained from urethral swabs in the male and cervical swabs in the female, were streaked onto Jembec plates. These plates were sent to the state laboratory for testing. In addition, two strains of gonorrhea, designated F62 and N9, were obtained from the Venereal Disease Research Laboratory at the Center for Disease Control (CDC).

Isolates were identified by the Gram stain, oxidase reaction and fermentation of sugars characteristic of N. gonorrhoeae.

B. Serum Samples

Once a presumptive positive test was established from culture samples, serum samples from the same patient were traced down from the syphilis serology laboratory. In the diagnosis of venereal disease in sexually active people, it is routine to check for both syphilis and gonorrhea. Sera from culture positive patients were labeled and frozen a -70 C until needed.

C. Isolation of Type 1 Colonies

Once a presumptive positive culture was found the bacteria were

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3 The majority of the samples used in this study came from the VD Clinic in Billings, Montana.

4 Jembec plates have the MTM medium and a CO₂ pellet to keep the CO₂ tension up.

5 I wish to thank Anne Sallgren for centrifuging the blood samples and saving the serum. I wish to also thank Vicki Archibald for helping me match serum samples and culture samples.
subcultured onto Bacto-GC medium base (GCBB) as stated by Kellogg et al. (20). This allowed for fast, efficient isolation of type 1 colonies to be used in the study as outlined by Juni and Heym (L9). Basically, this medium is a chocolate agar with Isovitalex and a minimum amount of hemoglobin. The near absence of hemoglobin results in a semi-opaque medium through which light can be transmitted. 6

The basis for the differentiation of the four main colony types rests on morphological differences between the colonies (29). T1 and T2 colonies are convex whereas T3 and T4 are relatively flat. Therefore, T1 and T2 can refract light and focus light while T3 and T4 can not focus the light as well.

An American Optical Corporation Model 569 Stereozoom dissecting microscope was used. The microscope was fitted with a substage mirror that was convex on one side and flat on the other. An improvement of the procedure outlined by Juni and Heym for adjusting the light and substage mirror for critical illumination was used. By loosening the set screw holding the lens assembly the housing was lifted off. One could then look directly (straight down) into the mirror and see exactly what Juni and Heym describe in length. First use the plane mirror and adjust the mirror so that the stage is reflected in the exact center of the mirror. Bring the light source (fluorescent lamp) close to the stage and adjust it so that light can be seen in the mirror above and below the reflected stage. This is the critical adjustment necessary for the light source, i.e. so that light will strike the colonies both from above and below (through the mirror) the stage. Once the light source has been adjusted, the convex mirror was used to observe the colonies because the plane mirror does not differentiate

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6A hearty thanks to Mrs. Donna Hoyt for preparing this special medium for me.
the colonies as well as the convex mirror. It will appear that the reflected stage takes up the entire convex mirror but the colonies will serve as tiny lenses and focus the stage in the colony. The lens assembly was put back on, the set screw tightened and the petri dish with colonies placed on the stage.

The stage, being focused by the colonies, appeared as a bar within the golden colored colonies at 30X magnification. The smallest colonies, approximately .5mm, were types 1 and 2 (the virulent types), while the larger colonies, 1.0 to 1.5mm were types 3 and 4 (nonvirulent). T1 and T2 colonies focused the stage, i.e. have a bar in the colony, whereas T3 and T4 did not. T1 colonies were further distinguished from T2 colonies in that T1 colonies had a bar with diffuse, fuzzy edges. This is because T2 colonies are nearly doubly convex and the edge of the colony meets the medium with a rounded edge (like a drop of mercury). T1 colonies are only singly convex (thus, can still focus a bar) and have edges which merge with the medium at a gentle slant. This caused the edges of the bar to appear more diffuse. In this study I was concerned with only isolating T1 colonies, but all types can be isolated following the procedure of Juní and Heym.

T1 colonies were picked off the media with a sterile needle and streaked onto another plate containing GCBB medium. Approximately three to four subcultures were necessary before plates were obtained that were 100% T1 colonies. The bacteria were then harvested in phosphate buffered saline (PBS). The pH was adjusted to 9. Suspensions of the bacteria

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7 Three cultures were subcultured ten times without a T1 colony appearing. Kellogg states that 90% of the primary isolates are T1 colonies. My own experience is that only 50-75% of the colonies are T1 after one subculture and in some cultures T1 colonies are absent.

8 Na₂HPO₄ .1M; pH 9. 14.2 g dissolved and brought to 1 liter with distilled water.
were made which matched the optical density of the MacFarland 0.5 sensitivity standard (23). These suspensions were frozen at −70 C until slides were to be made.

D. Conjugate

Fluorescein-labeled anti-human rabbit globulin conjugate was obtained from CDC for use in the experiment. The powdered conjugate was rehydrated with one ml of distilled water. One tenth ml aliquots of this conjugate were placed in each of ten vials. Nine ml of PBS containing two percent Tween-80 was added, effecting a 1;10 dilution (5). These vials were then frozen at −70 C until the tests were to be run.

E. The Immunofluorescent Antibody Test (24,27,35)

On the day of testing, the serum samples were placed in a 45 C water bath to thaw. Dilutions of the serum were made to determine at which dilution a positive test would result.

The frozen suspensions of the bacterial samples were then thawed. These suspensions yielded approximately 200–300 separate bacteria per high power field (450X) when dropped on the slide. Approximately .05 ml of the bacterial suspension was dropped in each well of specially made twelve-well slides. The slides were allowed to air dry. It was not advised that formalin-fixed bacteria be used because there is a loss of specificity of the antibody for the antigen. The bacteria, so fixed, served as antigen in the IFA test.

The serum dilutions were then dropped on the slides, each dilution to a different well. The slides were placed in a moist chamber and incubated at 37 C for thirty minutes. This allowed for antibody in

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9Cat. No. BC 1508 Lot 78-0101.

10It is important that the slides not dry out during incubation. A chamber with wet paper towels serves to keep the slides moist,
the serum to bind with the antigen found on the bacterial cell surface.

While the slides were incubating, one vial of frozen, 1:10 diluted conjugate was thawed. One tenth ml of conjugate was removed to another vial and 2.4 ml of PBS added. This 1:25 dilution plus the original 1:10 dilution brought the total dilution to 1:250. The vial was wrapped in paper to prevent light from quenching the fluorescein. Any unused thawed conjugate was discarded.

The slides were taken out of the incubator after one-half hour and rinsed twice in PBS. The slides were blotted and allowed to air dry. Conjugate was added using capillary tubes. The slides were placed in the moist chamber and incubated at 37°C for thirty minutes. After one-half hour, the slides were rinsed twice with PBS, blotted and allowed to air dry. One drop of Difco Bacto FA Mounting Fluid was added and a Corning Cover Glass No. 1 (22 mm sq.) coverslip was applied. The slides were then placed under paper towels to prevent quenching of the fluorescein (See flow chart in Fig. 4).

A Zeiss Darkfield Fluorescent microscope was used to read the slides (400X). The cells appeared as bright yellow spheres in a dark field. The filter was moved into position causing all the light to be absorbed except the light coming from the fluorescing cells, i.e. the cells which have antibody bound to them and fluorescein bound to the antibody.

The amount of fluorescence was subjectively determined and given a rating shown in Table 2. At least three to five high power fields from each well were selected for examination. Grading was based on the fluorescence of isolated bacteria only, since clumps of bacteria sometimes stained nonspecifically and made the scoring of fluorescence very difficult.

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11 CDC determined the conjugate dilution to be 1:300 but I used a 1:250 dilution because I wanted to be sure the conjugate would bind.
Fig. 4. Flow chart of IFA technique.

Antigen (Frozen Suspension of Bacteria)

- Thaw
- Drop on Twelve-Well Slides
- Air Dry

Serum

- Thaw
- Dilutions

Conjugate

- Thaw
- Dilute & Wrap

- Incubate 37 C
  30 Minutes in Moist Chamber
  Rinse 2X
  Blot & Air Dry

Mounting Fluid & Cover Slip Wrap

Read Slides
4+ = brilliant fluorescence of all organisms in the field

3+ = well defined fluorescence of all organisms in the field

2+ = low intensity, but definite fluorescence of seventy five percent or more of the organisms in the field

1+ = occasional organisms with low intensity fluorescence

± = doubtful fluorescence

− = absence of fluorescence, only a low grade background fluorescence or fluorescent clumps of gonococci seen with no evidence of fluorescence specifically associated with isolated organisms

Table 2. Grading Scale Used in Determining the Amount of Fluorescence.
When examining the bacteria, care was taken to look for peripheral accentuation of the stain and to note the contrast between the central and peripheral staining. In brightly fluorescing bacteria the contrast became less marked and the fluorescence appeared predominantly homogeneously stained.
RESULTS

A. Determination of Dilution Range

Initial experiments were designed to determine which dilution of serum would give a reactive test (2+ fluorescence or higher) and allowed me to become more proficient in using the IFA technique. Nine serum samples were tested against autogenous antigen (gonococci derived from patient) and the F62 and N9 antigens at dilutions which ranged from 1:1 to 1:2048. The results are shown in Table 3. The highest titer showing reactivity was 1:64 for autogenous and N9 antigens while 1:16 was the highest titer for F62 antigen. All dilutions past 1:64 showed no fluorescence of 2+ or higher. The mean titer for autogenous antigen was 1:16, the range was from 1:8 to 1:64. The mean titer using strain F62 as antigen was 1:8, while the range was from 1:4 to 1:16. The mean titer for strain N9 was 1:16; the range was from 1:2 to 1:64.

B. Determination of a Diagnostic Titer

These tests were designed to determine at which single titer could a positive reaction be considered diagnostic for gonorrhea. Nine serum samples were tested against strains F62 and N9, at dilutions which ranged from 1:2 to 1:64. The percent of the sera which showed reactivity at the various dilutions are tabulated (Table 4).

The reactivity of N9 dropped in equal intervals of approximately nineteen percentage points. Strain F62 showed the greatest drops in fluorescence from one dilution to the next with 31 and 25 percent drops between dilutions 1:8 and 1:16 respectively. Autogenous antigen showed the least drop in fluorescence as 37 percent of its sera were still reactive at 1:64 dilution.
<table>
<thead>
<tr>
<th>Antigen</th>
<th>1:2</th>
<th>1:4</th>
<th>1:8</th>
<th>1:16</th>
<th>1:32</th>
<th>1:64</th>
</tr>
</thead>
<tbody>
<tr>
<td>Autogenous</td>
<td>100</td>
<td>100</td>
<td>89</td>
<td>78</td>
<td>44</td>
<td>37</td>
</tr>
<tr>
<td>F62</td>
<td>100</td>
<td>94</td>
<td>75</td>
<td>44</td>
<td>19</td>
<td>6</td>
</tr>
<tr>
<td>N9</td>
<td>100</td>
<td>81</td>
<td>62</td>
<td>44</td>
<td>31</td>
<td>6</td>
</tr>
</tbody>
</table>

Table 4. The percent of sera from culture positive patients which show reactivity at the given dilution.
C. The Blind Test

Several serum samples from cultural positive and culture negative patients were coded and randomly selected by one person and then tested in a blind fashion by me. Only strains F62 and N9 were used as antigen. Each test sample was judged either positive or negative at dilutions of 1:8 and 1:16. Four possible results were possible upon decoding the data:

(1) One can predict a positive and a positive is culturally confirmed.
    = CORRECT

(2) One can predict a positive and a negative is culturally confirmed.
    = FALSE POSITIVE

(3) One can predict a negative and a negative is culturally confirmed.
    = CORRECT

(4) One can predict a negative and a positive is culturally confirmed.
    = FALSE NEGATIVE

The results are shown in Table 5.

Strain F62 at a dilution of 1:16 predicted 71% of the samples correct while N9 only predicted 30% of the samples correct. Neither strain showed much accuracy at a dilution of 1:8 in predicting gonococcal infection at which F62 predicted only 42% and N9 only 50% of the samples correct. N9 at a dilution of 1:8 had the highest percentage of false positive predictions while at a 1:16 dilution it had the highest percentage of false negative predictions.

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12 I wish to thank Mark Hegewald for coding and selecting my samples for me.
Table 5. Comparisons of the accuracy of F62 and N9 in predicting culturally-confirmed gonorrhea at titers of 1:8 and 1:16.

<table>
<thead>
<tr>
<th>Antigen</th>
<th>No. Tested</th>
<th>1:8 Titer</th>
<th>1:16 Titer</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>False Negative</td>
<td>False Positive</td>
</tr>
<tr>
<td></td>
<td></td>
<td>No.</td>
<td>%</td>
</tr>
<tr>
<td>F62</td>
<td>17</td>
<td>7</td>
<td>41</td>
</tr>
<tr>
<td>N9</td>
<td>18</td>
<td>9</td>
<td>50</td>
</tr>
</tbody>
</table>
DISCUSSION

A. Determination of Dilution Range

In this study, the accuracy of the IFA test in detecting gonococcal infection was investigated. To use the test it was necessary to first determine the range of dilutions within which one could observe a change in fluorescence from reactive to non-reactive. Although the mean dilution of 1:16 for strain N9 agrees with the results obtained by Welch and O'Reilly (35), the data presented here did not show reactivity past the 1:64 dilution, i.e. the range was more restricted. The range of dilutions from 1:2 to 1:64 was found to best reflect the change in fluorescence. This range was therefore used in the next set of tests.

In addition, strain F62 showed more reactivity, having a mean titer of 1:8 and not being reactive past a 1:16 dilution. In contrast, over one half of the N9s were reactive at a 1:32 dilution or greater. These data seem to not support a claim by CDC that F62 is more specific in binding gonococcal antibodies.

B. Determination of a Diagnostic Titer

An attempt was made to determine at which titer could a positive reaction be considered as diagnostic for gonococcal infection. Also, the different antigens were compared to see which one showed the best drop in fluorescence and could thus be a useful indicator of gonococcal infection. F62 and N9 served as better antigens than autogenous antigen because they showed a greater drop in the percent of sera that were reactive within the range of dilutions determined in part 1. Antigen N9 showed gradual drops in fluorescence (averaging 19 percent with each dilution) whereas F62 showed 31 and 25 percent drops in fluorescence between dilutions 1:8 and 1:16. It appeared that F62 was a better antigen from these results. However,
there was no sharp drop in fluorescence at any single dilution. It appeared that the 1:8 and 1:16 dilutions showed the greatest drops in fluorescence and seemed to be the most diagnostic. These two dilutions were used in the blind test.

C. The Blind Test

Once the aforementioned parameters had been determined, the blind test was set up to simulate a clinical laboratory analysis. In the blind test only strains F62 and N9 were used as antigen because (1) they showed the best drop in fluorescence within the range of dilutions and (2) if the IFA test was to be a serological test, cultures from the patients would not be available for use as antigen.

The 71 percent correct predictions of gonococcal infection by strain F62 at a dilution of 1:16 agrees well with the results obtained by Welch and O'Reilly.\textsuperscript{13} The major error came in predicting false negative results. Strain N9, on the other hand managed to predict only 50 percent of the cases correctly at a serum dilution of 1:16; it also showed a similar incidence of predicting false positive and false negative results. Clearly, F62 antigen appeared to be the best antigen to use in the IFA test and the 1:16 dilution seemed to be the best diagnostic titer.

D. Sources of Error

The IFA test can be a useful test for defining the course of the humoral immune response to gonococcal infections. However, its use as a model for a serological screening test for the presence of gonococcal infection must be tempered by the consideration of a number of factors.

First, there is a time period between onset of infection and the

\textsuperscript{13}Welch and O'Reilly correctly predicted 79 percent of the infected females and 52 percent of the infected males. My results were obtained using sera from male and female patients.
appearance of detectable antibody. If a serum sample is taken during this period a false negative prediction will result. Also, the antibody response will vary according to the type and degree of infection and the reactivity of the host. The duration of this time period may be anywhere from five to ten days or longer (10). In some cases, no detectable antibody response is ever seen by an infected patient. This may be because the infection is so localized that only a cell-mediated response occurs and there is no humoral antibody response.

Second, the duration of the antibody response itself is significant especially when studying patients with histories of recurrent of previously treated infections. The duration of serological response varies from one to six months (35). A treated person who is culturally negative may still have antibodies to the gonococcus in their serum and a false positive prediction would result. This fact is significant when screening a general population because a great number of patients who contract gonorrhea have have had it previously within the last six months.

Third, the characteristics of the antigen and the quality of the fluorescent conjugate play an important role in the IFA test. The use of media lacking the defined supplement can cause sudden loss of the organism's antigenicity.

Fourth, serotypes of N. gonorrhoeae peculiar to a certain geographic region may be very specific for the antibodies found in one patient but not very specific for the antibodies found in another patient infected by a different serotype. The strains F62 and N9 were purported to be very specific for antibodies against the types of gonorrhea isolated around Atlanta, Georgia, where CDC is located. They may have a lessened specificity for the serotypes found in Montana where all of my samples were
I did encounter problems in this study which may have contributed to a low accuracy of prediction. One problem was a changing intensity of the light source from the dark-field fluorescent microscope. The poor light source (discovered after the slides had been read) resulted in a very low intensity of fluorescence. This may be why I did not have any positive fluorescence past a dilution of 1:64 while other workers had reported fluorescence at 1:256 dilutions. Another problem was that I did not know the medical history of the patients, so could not tell if they had contracted the disease recently or if they were being tested for cure.

Areas for possible improvement of the IFA test include a continued search for more representative strains of gonococcus to serve as antigen, isolation of specific antigens which could be adsorbed to solid matrices and used fluoremetrically and a better understanding of the mechanisms of the humoral response in humans to gonococcal infection.

In conclusion, then, this study does propose an improved technique for the isolation of T1 colonies of N. gonorrhoeae. It also showed that even though the IFA test is not a good test for the presence of gonorrhea, it is a promising technique which has a number of advantages over culture confirmation. At present, the test needs a number of problems to be worked out before the technique can be used diagnostically in a clinical setting. With the number of cases of gonorrhea increasing drastically each year, the IFA test may prove helpful in curbing the No. 1 infectious disease in the United States.


5. ------------ Directions For Use of Fluorescein-Labeled Anti-Human Globulin.


