# DIRECT FA TECHNIQUE USING FLAZO ORANGE COUNTERSTAIN IN IDENTIFICATION OF NEISSERIA GONORRHOEAE

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IN THE immunofluorescent diagnosis of gonorrhea previously described (1–3), a direct and a delayed procedure for identifying gonococci in exudates have been used. When sufficient number of gonococci are present in the exudate, direct fluorescent antibody (FA) staining of smears permits detection and identification of the gonococci within less than an hour even when many contaminating bacteria are present.

When the direct FA method is used, the presence of only a few gonococci in an exudate results in a 50 percent loss of sensitivity compared with results by the delayed FA method (*1*). By the delayed FA method, exudate collected with a swab is incubated 16 to 20 hours on chocolate agar slants, and gonococci too few to detect by the direct FA technique grow to easily observable numbers. Rapid diagnosis of gonorrhea is often necessary, and the longer time required with the delayed FA method is undesirable.

When the simpler, faster, direct FA procedure is used, differentiation of specific *Neisseria gonorrhoeae* fluorescence is difficult even when the organisms are present in sufficient numbers to permit identification. Various counterstains have been reported to increase the effectiveness of the FA method (*4–6*). These counterstains allow faster and more specific detection of microorganisms in smears by increasing the background contrast.

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Careful attention to the collection of specimens for direct smears is necessary for the successful detection of gonococci. It has been suggested (*7*) that the use of a bacteriological loop is preferable to a swab for the collection of specimens from female gonorrheal patients, particularly when a small amount of exudate is present.

In this study the results of examination of smears by the direct FA (DFA) method and by the direct FA method using Flazo orange counterstain (FFA) were compared with the cultural results when specimens were obtained with a bacteriological loop.

## Materials and Methods

*Specimens.* Material for this study was obtained from 156 named female contacts of male gonorrheal patients. Specimens were taken at the Fulton County Health Department, Atlanta, Ga., by Dr. John H. Tiedemann, venereal disease control officer. The women were prepared as described in Public Health Service Publication No. 499 (*7*), care being taken to clean the external cervix. The speculum was then used to depress the cervix and strip the glands of secretion.

Two smears from secretion of each patient were prepared on microscope slides on which a 6 mm. circle had been etched. A 1 mm. loopful (28 gauge wire loop) of secretion was spread uniformly within the circle, air-dried, and gently heat-fixed. Smears were either stained immediately or stored at 5° C. for no longer than 5 days.

Exudate from the same patients was inoculated on Difco GC medium base enriched with

1 percent defined supplement as described by Kellogg and associates (*8*), modified by the addition of 0.5 mg. percent ferric nitrate, and incubated at 35° C. in a candle jar.

Cultures were examined after 24 and 48 hours for colonies of *N. gonorrhoeae*, which were confirmed by oxidase, gram stain, FA, and carbohydrate-fermentation techniques.

*Flazo orange counterstain.* Flazo orange [2-Naphthol, 1-(5-chloro-2-hydroxyphenylazo)-] was prepared according to Hall and Hansen (*6*). The sample of Flazo orange was obtained from Dr. P. Arne Hansen. Ten mg. of dye was dissolved in 2 ml. of N,N-dimethylformamide, and 10 ml. of chelating reagent was added slowly with agitation. The ingredients of the chelating reagent were N,N-dimethylformamide 50 ml., distilled water 20 ml., 0.1 molar aluminum chloride 10 ml., 1 molar acetic acid 10 ml. This solution was adjusted to pH 5.2 with 1 molar sodium hydroxide and made up to 100 ml. with distilled water.

Fluorescence of the stock solution was developed within 30 minutes to 1 hour at room temperature (23°–26° C.). The development of fluorescence was tested with a Mineralight Model SL 3660 (peak emission at 3,660 angstrom units). The stock solution was stored at −20° C. The stability of stock solutions of Flazo orange was tested for gross fluorescence with Mineralight once each 2 weeks or oftener, and direct FA smears were counterstained and examined for the quality of background fluorescence.

A working solution was prepared daily by adding 1 ml. of the stock solution to 199 ml. of chelating reagent.

*Staining procedure.* Fluorescein-labeled antigonococcal rabbit globulin was prepared as described by Deacon and associates (*1*). Human serum was added in equal parts to this conjugate to block nonspecific fluorescent staining of *Staphylococcus aureus* and of polymorphonuclear leucocytes (*9*). Both the conjugate and serum were stored at −20° C. A drop (0.04 ml.) of conjugate was added to each smear and incubated in a humid chamber for 30 minutes at 35° C. Smears were rinsed and soaked for 10 minutes in carbonate-bicarbonate buffer, pH 9.0. One smear was blotted and a cover slip mounted with a drop of glycerol-saline solution (9:1). The duplicate smear was counterstained with Flazo orange for 5 minutes at room temperature, rinsed, and soaked in carbonate-bicarbonate buffer, pH 9.0, for 1 minute. Excess buffer was drained from the slide, and a cover slip was mounted with glycerol-saline solution.

*Microscopy.* A darkfield microscope equipped with an Osram HBO–200 mercury vapor light source, a Schott BG–12 exciter filter, and a Corning 3387 barrier filter were used to determine fluorescence.

Smears were read in a systematic manner, from left to right, top to bottom, until the entire smear was read or until positive results could be recorded. Negative smears required from 3 to 4 minutes to read.

Preparation of material and reading of smears were accomplished with the assistance of Charles E. Cravens and Bobby G. Welch, microbiology laboratory technicians, Venereal Disease Branch.

## Results

The direct smear technique with counterstain compared favorably with the culture method in the yield of positive results.

| *Method* | *Positive findings* |
|---|---|
| Culture | 88 |
| Direct FA | 72 |
| Direct FA with Flazo orange | 85 |

Thus, by the culture method, 56 percent of the 156 female contacts were diagnosed positive; by the direct FA smear technique with Flazo orange, 54 percent. In other words, the culture method was only 3.5 percent more effective in detecting gonorrhea than the direct FA smear technique with counterstain. The addition of Flazo orange increased the efficiency of the direct FA smear by 15 percent.

The table shows the extent of agreement in findings among the three methods studied. Specimens from 53 of the women were positive to all tests; specimens from 50 were negative to all tests. Thus, in 66 percent there was complete agreement among the three tests. In 20 women, only one of the three tests was positive; in 12 of these 20, the positive test was the culture. Although the culture was the most efficient in detecting gonorrhea, both types of FA

**Agreement among three diagnostic methods in the identification of Neisseria gonorrhoeae in 156 female contacts**

| Results with direct smears | Culture positive | | Culture negative | |
|---|---|---|---|---|
| | Number | Percent | Number | Percent |
| DFA+FFA+-- | 53 | 60 | 10 | 15 |
| DFA−FFA+-- | 18 | 20 | 4 | 6 |
| DFA+FFA−-- | 5 | 6 | 4 | 6 |
| DFA−FFA−-- | 12 | 14 | 50 | 73 |
| Total----- | 88 | 100 | 68 | 100 |

DFA—direct fluorescent antibody. FFA—direct fluorescent antibody with Flazo orange.

smears were positive in 10 subjects when the culture was negative.

The effect of pH on the brilliance of fluorescence of *N. gonorrhoeae* in direct smears was investigated. Rinsing in higher pH favored the brilliance of fluorescein. When pH 7.2, pH 8.0, and pH 9.0 buffers were tested, greater brilliance was noted with pH 9.0 carbonate-bicarbonate buffer than with the phosphate buffers.

While good results were obtained with undiluted Flazo orange when applied to smears for 5 to 15 seconds, equal results were obtained by diluting the stock solution of dye and increasing the staining time. The best results in respect to fluorescence of the counterstain and convenience of handling were obtained with a 1:200 dilution of stock solution applied to the smears for 5 minutes.

When stained with Flazo orange, polymorphonuclear leucocytes and epithelial cells exhibited a deep pink fluorescence. Bacterial cells other than gonococci showed a light pink fluorescence.

After 6 months' storage of the stock solution of Flazo orange at −20° C., good fluorescence was noted with Mineralight; smears stained with a working dilution of stock solution exhibited good pink fluorescence within polymorphonuclear leucocytes and other cells, while the gonococci stained typically yellow-green.

## Discussion

Direct smear preparations previously have been considered unsatisfactory for the diagnosis of gonorrhea in women (*1*, *10*, *11*). In this study, however, the direct FA method without counterstain gave a high percentage of positives. Although only the exudate from the cervical glands was examined, it may be assumed that a higher degree of accuracy in the detection of *N. gonorrhoeae* from exudates of the Bartholin's and Skene's glands would be found if a loop were used in the collection of the specimen for direct smear preparations. In the past and at present, both swab and loop have been suggested devices for specimen acquisition. Yet, for a variety of reasons, a swab is commonly employed. A swab is both a sponge and a trap for any exudate. The possibility of losing a small number of micro-organisms in a swab and, thus, obtaining a negative result should preclude its use. A loop does not present these difficulties if properly applied by a physician.

The use of Flazo orange in this study increased the number of positive direct FA smears to within 2 percent of the cultural results. Since Flazo orange is a fluorescent dye, all tissue blood cells and micro-organisms can be noted by the microscopist. Thus, he has points of reference in respect to specific staining. Moreover, counterstain smears can be read with much greater ease; reading time is reduced and eye strain lessened.

## Summary

Laboratory diagnosis of gram-stained cervical smears from women suspected of having gonorrhea was so unproductive in the past that when the culture method was used the stained smear procedure was not. Even after immunofluorescent methods had greatly improved diagnosis of direct smears, results were still about 50 percent inferior to those obtained with the delayed fluorescent antibody (FA) procedure or with the culture method. Often there are too few organisms in the direct smear to observe; also, some nonspecific staining of the background material occurs.

In the current study, by the use of Flazo orange as a counterstain, nonspecific background fluorescence was quenched without obscuring points of reference, such as pus cells. Eye strain was notably reduced. One hundred fifty-six female contacts of male gonorrheal

patients were examined; material for smears and cultures was taken with a bacteriological loop instead of the usual swab. Findings on 56 percent of the 156 women were positive by culture; findings on 54 percent were positive by FA smear with Flazo orange counterstain. Thus, in the more rapid FA procedure the method of smear preparation and counterstain resulted in specific diagnosis of gonorrhea to within 2 percent of results by the culture method.


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## Phenylketonuria Detection

Phenylketonuria was detected in 1 of every 10,000 infants tested by the inhibition assay method in a 29-State investigation during 1962–63. Among the 400,000 infants tested in 505 hospitals, 39 cases were found. The study was supported by the Children's Bureau of the Welfare Administration, Department of Health, Education, and Welfare. Previously it had been estimated that the disease occurred once in every 20,000 births, or as infrequently as once in every 40,000 births.

Another unexpected finding was a case of phenylketonuria in a Negro infant. Scientists had thought that this metabolic error did not occur in Negroes.

The Children's Bureau is encouraging States to insure the testing of all newborn infants on a routine basis. New York, Rhode Island, Massachusetts, and Louisiana have already passed laws requiring the inhibition assay test. Illinois, Oregon, and Minnesota have adopted the test pattern through permissive legislation or as a matter of public policy.

# Federal Publications

**Cancer of the Lung.** *PHS Publication No. 1173; 1964; 8 pages; 10 cents, $5 per 100.* Discusses symptoms, diagnosis, treatment, prevention, and the nature of cancer in general. Includes a section on smoking which describes some of the laboratory evidence and statistical studies that led the Surgeon General's Advisory Committee on Smoking and Health to conclude that the effect of cigarette smoking far outweighs all other factors related to lung cancer in men and that data for women, though less extensive, point in the same direction.

**Strictly for Teenagers.** *PHS Publication No. 913; adapted from Ohio Department of Health leaflet of same name; 8 pages; revised 1964; 5 cents.* Discusses venereal diseases frankly—tells what they are, how they spread, and the possibilities for cure. Answers many questions modern teenagers ask about this topic and corrects several misconceptions about these diseases. Challenges teenages to develop standards and attitudes that will guide their conduct and contribute to the prevention of venereal diseases.

**Civil Defense Emergency Hospital, Model-62, Component Listing and Storage Data.** *PHS Publication No. 1071–F–11; 1964; 40 pages.* Provides separate alphabetical listings for 14 categories of components comprising the completely functional 200-bed model-62 civil defense emergency hospital. Also provides the case number, Federal stock number, storage code, nomenclature, unit of issue, total quantity, total weight, and total cube by quantity of the 650 line items packed in 660 cases. These data enable warehousemen to position stockage appropriately for required Federal team inspection, special storage requirements for temperature, humidity, and fire hazard, replacements governed by expiration dates, and refurbishment of damaged items. Furnishes the State-appointed custodian with an additional aid to fulfill efficiently his detailed responsibilities in caring for prepositioned CDEH.

**Directory of State and Territorial Health Authorities.** *PHS Publication No. 75; 1964 revised; 104 pages; 35 cents.* Lists health department personnel of each State and Territory to reflect the organization of the department. Lists health department officials and all State and Territorial health officers, showing title, headquarters address, area code, and telephone number of each health department. Includes similar information for State agencies other than health departments administering grant programs of the Public Health Service and the crippled children's grant program of the Children's Bureau.

**Hospital Profiles: A decade of change, 1953–62.** *PHS Publication No. 930–C–7; 1964; 21 pages; 25 cents.* Reports results of studies of hospitals during the 10-year period, 1953–62. Part I presents information on distribution and utilization of all hospitals, and part II is concerned with non-Federal short-term general and special hospitals. Supersedes and updates material contained in "Prototype Study: Hospital operations and activities," and compiles data about current hospital operations and activities.

**Labor Standards for Public Health Service Construction Grant Programs for Hospital and Related Medical Facilities.** *PHS Publication No. 930–A–5; 1964; 14 pages.* Outlines the labor standards required in the following Public Health Service construction grant programs: hospital and medical facilities survey and construction (Hill-Burton); hospital and medical facilities under accelerated public works; facilities for the mentally retarded; community mental health centers; health professions teaching facilities; university-affiliated facilities for the mentally retarded; health research facilities; and centers for research on mental retardation and related aspects of human development. The publication includes the applicable provisions of the Davis-Bacon Act, the Contract Work Hours Standard Act, and the Copeland Act.

**Mental Retardation Program of the Division of Chronic Diseases.** *PHS Publication No. 1194; 1964; pamphlet.* Describes program of the new Mental Retardation Branch of the Division of Chronic Diseases, including planning grants to States, grants to agencies or individuals for professional and technical training, community serivce projects, and applied research. Designed primarily for professional personnel in the health, education, and welfare fields.

**Milk and Milk Product Equipment. A guide for evaluating sanitary construction.** *PHS Publication No. 1216; 22 pages; 15 cents.* Designed to assist milk regulatory officials in evaluating milk and milk product equipment for compliance with the sanitary ordinances and codes relating to milk and frozen desserts developed by the Public Health Service.

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**The Public Health Service does not supply publications other than its own.**

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PHR SYNOPSES

**CONSTANTINE, DENNY G. (Public Health Service), and WOODALL, DORA F.:** ***Latent infection of Rio Bravo virus in salivary glands of bats. Public Health Reports, Vol. 79, December 1964, pp. 1033–1039.***

Rio Bravo virus infection rates in 1,075 adult Mexican free-tailed bats and 100 cave myotis bats examined in this study varied from none, where the bats regularly inhabited ventilated roosts, to 3.4 percent, where they regularly inhabited extremely ill-ventilated roosts. Mixed groups of adult migrants had infection rates as high as 7 percent. The virus was absent in bats 2 weeks of age except for one bat, which was positive in lungs only. Otherwise, the virus was not recovered from brains, mammary glands, lungs, kidneys, or fetuses. Young bats were found to be infected when they were about 2½ months old.

A captive bat that appeared normal was infected throughout a period of 682 days, after which it was killed. Other infected captives, held for prolonged periods, failed to evidence symptoms. Two bats had the virus in salivary glands when captured, but their saliva eventually became negative, and virus was not isolated from salivary glands when the bats were killed 404 and 681 days after capture.

Although Rio Bravo virus was isolated from symptomatic bats in nature, the infected bats comprised a proportion of symptomatic bats no greater than their proportion in asymptomatic bats.

**WALL, M. A. (Public Health Service):** ***Application of statistical techniques to environmental radiation surveillance. Public Health Reports, Vol. 79, December 1964, pp. 1057–1064.***

The accurate measurement of radioactivity in environmental media depends ultimately on the validity of the counting procedures. The application of statistical techniques to the testing of such nuclear measurements is discussed. Reviewed are the importance of the randomness of the sample, the calculation of counting variability from the Poisson and normal distributions, the use of the chi-square statistics to compare the observed and theoretical variabilities, and the construction and use of control charts for routine operations. An example is given of the application of these concepts to the evaluation and control of the background counting rate of detection equipment used in the assessment of radioactivity levels in environmental samples and to the establishment of a quality control program which provides for the duplicate analysis of randomly selected samples from a variety of nationwide surveillance networks.

**GRAY, DAVID H. (State of Vermont Department of Health), STOWE, HAROLD W., and HOLDEN, ROBERT A.:** ***Rapid automated micro screening for diabetes. Public Health Reports, Vol. 79, December 1964, pp. 1081–1086.***

An adaptation of standard AutoAnalyzer methodology for micro blood glucose determinations using recent equipment modifications and a sample of 20 microliters of capillary blood was tested. A sampling rate of 60 determinations per hour was used. The blood sample was collected with the Unopette, a recently developed, disposable capillary pipette, delivering 20 microliters of blood into a collecting reservoir containing 1.3 ml. of a 1 percent sodium fluoride solution. Studies determined that the technique demonstrated sufficient rapidity, specificity, simplicity, and economy to recommend it as a tool for diabetes screening of large population groups. Cost analysis showed that each micro blood sugar determination cost approximately 7.8 cents per test.

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# SYNOPSES

**FRAUMENI, JOSEPH F., JR. (Public Health Service), and WAGONER, JOSEPH K.: *Changing sex differentials in leukemia. Public Health Reports, Vol. 79, December 1964, pp. 1093–1100.***

The continuous increases in U.S. leukemia mortality since 1921 were found in this study to be associated with sex ratios (male-to-female death rates) which have declined among children and increased among adults. The shifts in sex ratio occurred only in the white population and were demonstrated in leukemia statistics for England and Wales. To evaluate these changes, a comparison was made of U.S. white males and females according to the "relative" and "absolute" increases which have occurred in age-specific leukemia death rates. In each age group the direction of the changing sex ratios reflected the excess contributed by either females (in children) or males (in adults) to the relative or percentage increases in mortality. Of greater significance were trends produced by the absolute increments, which contained an approximately equal number of males and females in the childhood age groups and an increasing preponderance of males in each adult category. These trends suggest that (*a*) the rise in mortality from childhood leukemia has been caused either by leukemogenic factors introduced into the environment since 1921 and affecting both sexes equally, or by improvements in ascertainment of the disease; and (*b*) the rise in leukemia among adults has been real and not primarily related to improved ascertainment, with males selectively affected by increasing leukemogens in the environment.

**CHAPMAN, W. MAX (California State Department of Public Health), and MERRILL, MALCOLM H.: *Use and care of laboratory animals. California's law. Public Health Reports, Vol. 79, December 1964, pp. 1107–1111.***

A law relating to the use and care of laboratory animals has been constructively administered by the California State Department of Public Health for more than 12 years. It was sponsored by leaders in medical and other science education and by those responsible for laboratory animal care.

The law has been tested in the courts and found to be sound and workable. The general level of animal care has improved, and this is expected to continue. The cost of administration is covered partly by license fees and partly by the department's service budget.

Consideration of this experience may be useful to other States. An expansion of this principle in care of laboratory animals may possibly eliminate the need for Congressional action.

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SYNOPSES

**PEDERSEN, A. H. B. (Seattle-King County, Wash., Department of Public Health):** ***Cytological screening for cancer in a venereal disease program. Public Health Reports, Vol. 79, December 1964, pp. 1112–1118.***

In a cervical-uterine cancer screening program limited to women admitted to service in a venereal disease clinic in Seattle-King County, Wash., 1,885 women with a median age of 20 through 24 years were examined in a 2-year period. Definitive biopsy diagnosis and treatment as needed were offered to all patients showing class III or IV (suspicious or positive) cytological slides. The majority of patients were medically indigent and had not had a previous opportunity for this type of medical screening.

The median age of patients screened was about 24 years. A total of 11 cases (6.5 per 1,000) of carcinoma of the cervix were successfully brought to treatment. All but one of these cases were in the in-situ stage. An additional 5 patients among 36 who needed further tests but were lost from study presumably also had cancer of the cervix. If one adds these to the 11 patients brought to treatment, the presumptive prevalence rate would rise to 8.5 per 1,000.

The mobility of venereal disease patients makes followup difficult. Also, in many patients the presence of vaginitis and cervicitis from one cause or another complicates interpretation of their cytological slides. Yet an active venereal disease clinic, using its existing facilities and personnel, can probably screen these patients at little additional cost and with a substantial yield of early cases of cancer. Arrangements for adequate surgical followup should be made before undertaking such a screening program.

**PEACOCK, WILLIAM L., Jr. (Public Health Service), and THAYER, JAMES D.:** ***Direct FA technique using Flazo orange counterstain in identification of Neisseria gonorrhoeae. Public Health Reports, Vol. 79, December 1964, pp. 1119–1122.***

Laboratory diagnosis of gram-stained cervical smears from women suspected of having gonorrhea was so unproductive in the past that when the culture method was used the stained smear procedure was not. Even after immunofluorescent methods had greatly improved diagnosis of direct smears, results were still about 50 percent inferior to those obtained with the delayed fluorescent antibody (FA) procedure or with the culture method. Often there are too few organisms in the direct smear to observe; also, some nonspecific staining of the background material occurs.

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