A New Rapid Immunofluorescent Staining Technique for Identification of *Treponema pallidum* and *Neisseria gonorrhoeae*. (29090)

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The usual fluorescent antibody staining procedure for direct identification of microorganisms requires, among other items, a moist chamber, an incubator and a performance time of about one hour. This report describes a staining modification developed initially because of problems encountered in staining fresh *T. pallidum*. It allows for completion of the staining in less than one minute with results equal to or better than those obtained with currently available techniques. Findings in respect to *T. pallidum* suggest an important area of possible application.

Materials and methods. Smears from males with acute gonococcal urethritis and from female contacts were supplied by a local health clinic.* Smears from syphilitic and non-syphilitic lesions were collected by the Houston City Health Dept., Houston, Texas, and shipped to the laboratory *via* air mail.† Fresh isolates of *N. gonorrhoeae* were separated into colony types (T1-virulent, T2, T3 and T4-avirulent) and maintained by selective transfer(1). Cultures of other *Neisseria* and treponemes were available from laboratory stocks. Fluorescein-labeled antiserum against formalin-killed *N. gonorrhoeae* T4 cells was produced as described by Deacon(2). Conjugates against live T1 cells were made from antiserums produced by injecting live T1 cells (10 × MacFarland No. 10 density) twice a week for 3 weeks intravenously into rabbits which were bled from the heart 4 days after the last injection. Fluorescein-labeled treponemal antisera were produced according to the methods previously described by Deacon(3). The comparative FA staining procedures consisted of: 1) The usual direct method in which microorganisms are fixed to microscope slides by heating. After covering smears with fluorescein-conjugated antiserums, they are placed in a moist chamber for a period of 30-60 minutes at 37°C. Smear preparations are then rinsed in buffered saline (pH 7.2) for 5-10 minutes followed by a distilled water rinse and are finally mounted under glycerol-buffered saline and a cover slip. 2) The rapid immunofluorescent staining (RIS) technique in which a 6 mm diameter circle is cut in the fixed smear with a diamond point pencil in order to define the area to be stained by a 2 mm loop (28 gauge) of the conjugate. The conjugate is dried onto the smear at 45°C (25, 35 or 45°C does not influence results of the final preparation with either undiluted or diluted conjugates). The slide is rinsed under either running tap water, buffered saline or distilled water for 5 seconds and is finally mounted as described before.

Results. An equal number of *N. gonorrhoeae* cells stain with essentially the same brilliance by the RIS technique (3-4+) as with the usual procedure (4+). Between the 4 colonial types of *N. gonorrhoeae,* there is no difference in degree of staining. *N. meningitidis* demonstrates differential staining as seen in Table I depending upon the condition of the cells and the type of *N. gonorrhoeae* conjugate (T1, T2, T3 or T4) employed.

By the RIS technique, *N. gonorrhoeae* conjugates did not stain *N. catarrhalis* (3 strains), *N. subflava, N. flavescens* (2 strains each), *N. perflava* (2 strains), *N. sicca* (2 strains), *N. flava* (2 strains) and *N. hemolysans*. In addition, a variety of yeasts, true fungi, gram-negative and positive cocci and rods were not observed to stain by this procedure.

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TABLE I. FA Staining of Meningococci by *N. gonorrhoeae* Conjugates Using the RIS Technique.*

N. meningitidis Types		Live T1 conjugate	Formalinized T4 conjugate
Live	A	—	2–3+
	B	—	—
	C	—	2–3+
	D	—	—
Formalin-killed	A	2–3+	–to 1+
	B	2–3+	–to 1+
	C	2–3+	–to 1+
	D	2–3+	–to 1+

* *N. gonorrhoeae* 4+ with T1 or T4 conjugate.

N. gonorrhoeae are successfully stained in smears obtained directly from both males and females with gonorrhea and the stained smear is similar in overall appearance to smears stained by the usual procedure.

There is a degree of specificity inherent in the RIS technique itself when used for treponemal study (Table II). All treponemes except *T. pallidum* were stained less with the RIS technique than with the usual technique in spite of the use of conjugates with similar dilution factors. The decrease is clearly seen with *Borrelia vincentii* and the unabsorbed conjugate. The relatively constant staining of *T. pallidum* might indicate that such staining is for the most part due to specific antibodies. Support is lent to such a hypothesis by elimination of the staining of the Reiter treponeme and *B. vincentii* through Reiter absorptions of the conjugate as seen in the left hand column of Table II.

Direct smears obtained from human syphilitic and non-syphilitic lesions when stained by the RIS technique demonstrated results equal to those seen in Table II; that is, treponemes obtained from true chancres and presumed to be *T. pallidum* gave brilliant and clear-cut results. Spirochetes obtained from non-syphilitic lesions and presumed to be other than *T. pallidum* did not stain by the RIS technique and therefore demonstrated clear-cut differentiation.

TABLE II. FA Staining* of Treponemes by the RIS and the Usual Techniques.

Treponemes	Reiter treponeme absorbed *T. pallidum* conjugates		Unabsorbed *T. pallidum* conjugates	
	RIS	Usual	RIS	Usual
T. pallidum	2–3+	2–3+	3+	3–4+
T. pertenue	1–2+	2–3+	2–3+	3–4+
Reiter	—	±	1–2+	2–3+
B. vincentii	—	±	1+	3+
T. microdentium	—	—	—	±

* 1–2+ indicates individual treponemes may stain either 1+ or 2+.

Discussion. The basis of the operation of FA staining by the RIS technique is unknown. The rapidity of the actual staining by drying (30 seconds at 45°C) indicates that the rate of fluorescent antibody binding by the antigen is comparable with some other antigen-antibody systems(4). This rate is higher than that observed by the usual technique with either *N. gonorrhoeae* or *T. pallidum* or most other antigen-antibody systems. It is quite likely that temperature, rapidly increasing concentrations of antibody about the foci of bacteria during drying and antibody specificity are all influential factors in the operation of staining by the RIS technique. The specificity of the staining is shown by the inhibition of the specific staining of either *N. gonorrhoeae* or *T. pallidum* cells by their respective unlabeled specific antibodies. Where non-specific staining of background is a problem, normal human serum is effective in reducing this staining when mixed with the specific conjugate due to the presence in both conjugated rabbit serum and unconjugated human serum of antibodies against antigens common to a variety of life forms (5). Such a one-step inhibition is particularly effective during staining of smears directly from patients.

The 3 main advantages of the RIS technique are speed, simplicity and specificity without loss in sensitivity. Most important from the standpoint of future application and need is the ability of this new technique specifically to identify *T. pallidum* directly in smears from syphilitic lesions. At present an identification of *T. pallidum* can be made by either darkfield examination or by an indirect FA procedure(6). The darkfield findings, based on characteristic morphology and motility are subjective data in that insufficient numbers, lack of motility, mixtures of

treponemes and numerous artifacts are factors which can make interpretation difficult. The indirect FA procedure is time-consuming and employs a non-specific antibody. Neither of these two procedures can detect *T. pallidum* present as a minority in a mixture of treponemes. The RIS technique specifically stains *T. pallidum* in smears from syphilitic lesions in less time than would be required for making a gram stain preparation of other microorganisms.

These results indicate that a further evaluation of this process should be made. Confirmation of our conclusions may allow smears to be collected at distant points and shipped to central laboratories for rapid and specific diagnosis.

Summary. An immunofluorescent staining technique has been devoloped which specifically stains *T. pallidum* or *N. gonorrhoeae* in less than one minute. Application of the technique to direct smears of either organism has demonstrated its practicability as a diagnostic tool.

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Characteristics of Five Rhesus Monkey Kidney Cell Lines. (29091)

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The monkey kidney tissue cultures commonly used in studies of viruses are primary: Monkeys are killed and cells from their kidneys are cultured usually on a glass surface for 3 to 12 days. The cultures are then inoculated with viruses, or control materials. Such a procedure has several drawbacks, including the following: (a) The monkey colony is often seriously depleted, especially when the purposes of the virus research require thousands of such cultures. (b) The cultures themselves often carry viruses.

From September 1961 to January 1962, we tried to establish monkey kidney cell lines with 24 batches of cells from rhesus monkeys *(Macaca mulatta)* and 3 batches from cynomolgus monkeys *(M. irus)*. These batches were among those routinely supplied in our laboratories for investigations of the enteroviruses and certain other viruses. Five of these 27 attempts succeeded. In this report we describe the distinctive morphological features of these 5 lines, compare them with primary cultures in susceptibility to intact "wild-type" polioviruses and to ribonucleic acid (RNA) therefrom, and characterize 8 poliovirus mutants selected through passages in these lines.

A note on the RNA susceptibility of these lines appeared earlier(1).

Materials and methods. Primary cultures. The primary cultures were prepared in our laboratory by a standard procedure: Diced kidneys from rhesus or cynomolgus monkeys were digested with trypsin to obtain cell suspensions, which were finally suspended either in lactalbumin growth (LG) medium(2) or in medium S, which is high-cystine altered Eagle's maintenance ($hcAE_m$) medium(3) containing 4% calf serum. Usually 2-5 monkeys were used in the preparation of each batch of cells. The calf serum used in the