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An evaluation of fluorescein labeled monoclonal antibodies in the diagnosis of chlamydial infection

Author
Lipkin, Ellen,

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AN EVALUATION OF FLUORESCEIN LABELED MONOCLONAL ANTIBODIES IN THE DIAGNOSIS OF CHLAMYDIAL INFECTION

by

ELLEN LIPKIN

THESIS

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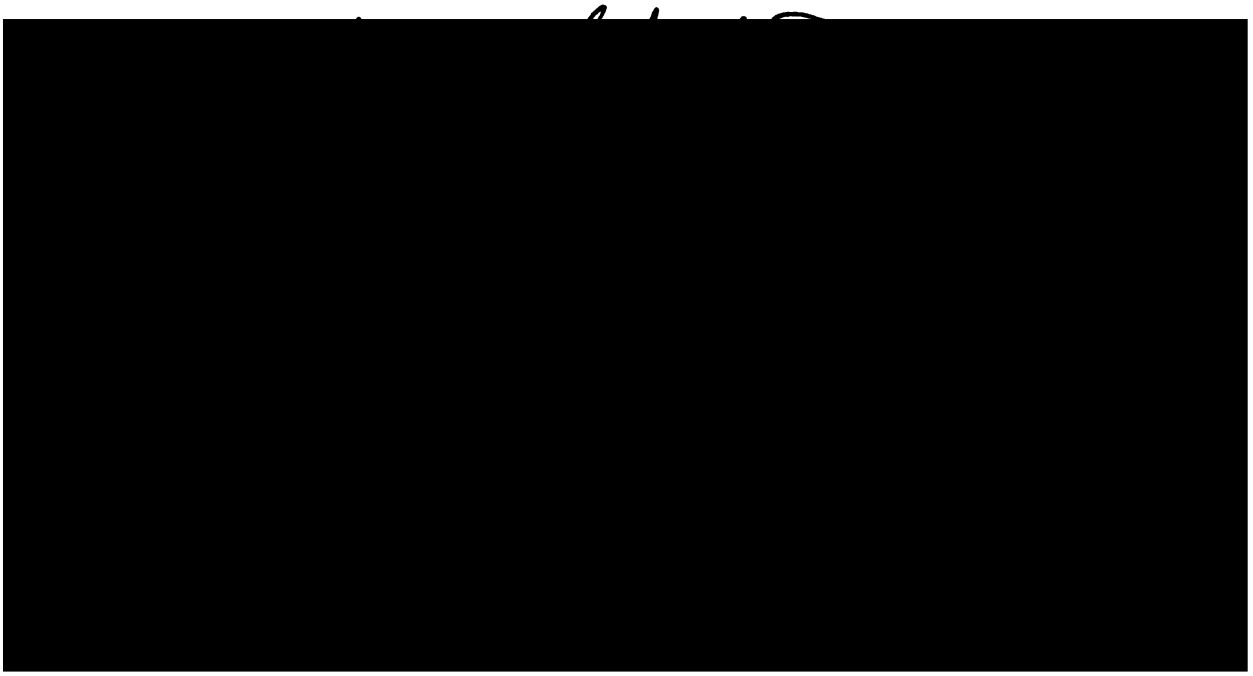

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INTRODUCTION

Chlamydiae are highly specialized Gram-negative bacteria that share a unique growth cycle which serves to differentiate them from all other microorganisms. They are obligate intracellular parasites which infect and subsequently multiply in susceptible cells (1,2). The chlamydiae are composed of a single genus Chlamydia with two species C. trachomatis and C. psittaci. Both species are capable of causing a broad spectrum of disease and are widespread in nature. C. psittaci is a common pathogen of lower mammals and birds and is the cause of human psittacosis, which may manifest itself either as a respiratory or a systemic disease. Psittacosis is contracted through exposure to discharges of infected avian species and is recognized as a serious occupational hazard to people in the poultry processing and pet bird industries. Whereas C. psittaci infect humans only as a zoonosis, the C. trachomatis strains are human pathogens with man as the major recognized natural host (with the exception of a few strains of rodent pneumonitis) (3,4). Human diseases caused by C. trachomatis include inclusion conjunctivitis, trachoma (4), urethritis (5), cervicitis, salpingitis (6), epididymitis (7), proctitis (8), lymphogranuloma venereum (3), and neonatal pneumonia (9, 10). It has also been implicated as a potential cause of Reiter's Syndrome (11) and most recently in pharyngitis (12). In developing

nations, trachoma, the world's leading cause of preventable blindness, effects 500 million people and is passed from child to child. In industrialized nations, most transmission of chlamydial infection is through sexual contact or to the infant during passage through the birth canal (4). The significance of sexual transmission in the spread of disease in industrialized countries has been recognized in the last decade. With an estimated 3 to 10 million new C. trachomatis infections annually in the United States, the prevalence of chlamydial infection is thought to exceed that of gonorrhea (13-15), thus making it the most common venereal disease.

Chlamydiae are restricted to intracellular parasitism because they are incapable of synthesizing ATP and thus may be considered energy parasites. The complex development cycle of chlamydiae is not yet fully understood. The length of the cycle is approximately 48 hours, but can vary as a function of the infecting strain, cell type and temperature (3). In naturally occurring chlamydial infections, there appear to be some differences in the ability of chlamydial strains to infect different cell types. C. trachomatis strains (other than LGV) appear to infect columnar epithelial cells, and most diseases they cause reflect transmission along the mucous membrane surfaces containing these susceptible cells. C. psittaci strains, on the other hand, appear to have a broader host cell spectrum and are

often found in macrophages (2).

There are two forms of chlamydiae which are involved in the growth cycle: the elementary body, the smaller, infectious form (~200-400 nm) and the larger, initial or reticulate body (~800-1000 nm) which is the metabolically active, replicative intracellular form. The chlamydial growth cycle involves the attachment of an infectious elementary body (EB) onto the surface of the host cell. The cell then actively phagocytizes the attached particle into the phagocytic vesicle. These endocytosed chlamydiae possess the ability to inhibit lysosomal fusion with the enclosing vesicle (2). The small EB then undergoes reorganization, such that in approximately 6-8 hours, it has changed from an EB to a reticulate body (RB). The metabolically active RB multiplies by binary fission until approximately 18-24 hours after infection. At this time, the RBs stop multiplying and undergo another reorganization during which they "condense" to form smaller EBs. These elementary bodies are then released from the inclusion and can infect other cells, thus repeating the cycle. The mechanism of release of EBs from cells is not understood (1-4,16,17).

Chlamydiae have known by a variety of names, the most important being Bedsonia and Miyagawanella. In 1966, much of this confusion was largely resolved by Page who suggested that the genus Chlamydia be used for the psittacosis-

lymphogranuloma venereum-trachoma group of organisms. In 1968 Page proposed that 2 species be recognized within the genus: C. trachomatis and C. psittaci (17). The proposal to establish a separate order of Chlamydiales was formalized by Storz and Page in 1971. There has been additional taxonomic confusion associated with chlamydiae. Inclusions of the trachoma agent in conjunctival scrapings were first observed in 1907 by Halberstaedter and von Prowazek, who thought they were protozoans and who proposed the term Chlamydozoaceae (Grk:cloak) for these "mantled animals" (a misnomer). They were later called large viruses when they were associated with the psittacosis outbreak of 1929 and recognized as filterable agents that grow in living cells. Once the concepts of R.Y. Stanier and A. Lwoff on the fundamental differences between bacteria and viruses had been formulated and generally accepted, it became clear that chlamydiae are bacteria. Unlike viruses, chlamydiae possess both types of nucleic acid, DNA and RNA, multiply by binary fission rather than by self assembly, possess a discrete cell wall, analogous in structure and content to Gram negative bacteria and are susceptible to antibiotics. The sole feature that chlamydiae share with viruses is the obligatory intracellular nature of their parasitism and their mutual lack of independent energy production (4). They are also distinguished from the other group of intracellular bacteria, the rickettsiae, by their inability

to synthesize compounds for high energy storage, their lack of preferential use of glutamate, their lack of cytochromes and by their developmental cycle.

Both species include a wide variety of microorganisms with different biologic and serologic properties (3). C. trachomatis includes the mouse pneumonitis strain, the lymphogranuloma venereum strains (LGV), (serovars L-1, L-2, L-3) and the trachoma-inclusion conjunctivitis strains (TRIC), (serovars A-K). Of these, A, B, Ba, and C serovars have been regularly associated with trachoma, whereas serovars D-K have been associated with sexually transmitted infections and sporadic cases of conjunctivitis. Thus it seems likely that there are at least 3 species included in the current designated species of C. trachomatis. C. psittaci also include a large number of serovars and possess different biological properties (1).

The two species are differentiated on the basis of sulfonamide susceptibility and glycogen accumulation (or iodine staining) within the inclusion. C. psittaci strains are sulfonamide resistant and iodine stain negative, whereas C. trachomatis strains are susceptible to sulfonamides and their inclusions stain with iodine (2).

The actual number of C. trachomatis infections is unknown (since they are not reportable conditions), but genital tract disease due to C. trachomatis infection is now considered a major health problem. A summary of sexually

transmitted diseases caused by C. trachomatis is shown below.

Table 1. Sexually Transmissible Diseases Caused By C. trachomatis (19)

Men and Women	Women
Lymphogranuloma Venereum	Cervicitis
Inclusion Conjunctivitis	Salpingitis
Otitis Media	Urethritis-Urethral Syndrome
Proctitis	Bartholinitis
Pharyngitis*	Perihepatitis
	Endometritis
Men	**Infants**
Urethritis	Inclusion Conjunctivitis
Epididymitis	Pneumonia
Reiter's Syndrome*	Rhinitis
	Otitis Media
	Vaginitis*

*suspected

The clinical spectrum of disease associated with C. trachomatis parallels that of N. gonorrhoeae. There are also similar epidemiologic patterns for these two organisms (20,21), with the same groups identified as high risk (i.e., sexually active teenagers, particularly from economically disadvantaged groups). The two infections often occur

together; about 20% of males and 40% of females with gonococcal infection have concomitant chlamydial infection (3). In most settings other than urban center VD clinics, chlamydial infection is more common than gonococcal infection (22). For example, routine screening of women attending family planning clinics found chlamydial infection rates to be 5-10 times those observed for gonococci (23).

The primary symptom of chlamydial infection in the male is urethritis. Approximately 40% of nongonococcal urethritis (NGU) and 80% of postgonococcal urethritis (PGU) are caused by C. trachomatis (24). PGU occurs after treatment of gonorrhea and is a consequence of coinfection with Chlamydia, which is not susceptible to penicillin. A serious complication of chlamydial infection in males is epididymitis (7). More than 250,000 cases of epididymitis each year are caused by C. trachomatis in the United States. C. trachomatis also causes proctitis in homosexual males (8) and, although seldom seen in the United States, lymphogranuloma venereum is caused by specific serovars of Chlamydia (3).

In the female, most chlamydial infections involve the cervix. The infection may be clinically inapparent or may result in a severe cervicitis. C. trachomatis infection is often asymptomatic. Consequently, treatment is not sought and the infected female is subject to serious complications, the most important being salpingitis.

Approximately 20-30% of the acute salpingitis seen in the United States is due to Chlamydia (13). In Scandinavian countries, where gonococcal infection has decreased in incidence overall, the percentage of acute salpingitis due to Chlamydia is higher (25). If salpingitis is not treated or is inadequately treated, the consequences could be infertility due to fallopian tube obstruction, ectopic pregnancy, or chronic pelvic pain. Of the estimated 200,000 women who develop chlamydial salpingitis in the United States every year, approximately 20,000 (10%) become infertile and 3,600 suffer ectopic pregnancy (13). Cervical infection in pregnant women has also been implicated in prematurity and neonatal death (26).

The asymptomatic female may also unknowingly infect her sexual partner and her newborn infant. Approximately one-third to one-half of exposed infants develop neonatal inclusion conjunctivitis and 10-20% develop pneumonia (11). Adult inclusion conjunctivitis (an acute follicular conjunctivitis) is also sexually transmitted when C. trachomatis infected genital tract discharges reach the eye (either by autoinoculation or during sexual activity).

In lieu of laboratory diagnostic procedures to demonstrate chlamydiae (which in the past have entailed tissue culture, thus necessitating costly facilities not generally available and requiring 3 to 7 days for

completion), presumptive indicators have been used in diagnosing chlamydial infection. The clinician most often establishes a diagnosis of NGU by excluding gonorrhea. The patient is then treated with tetracycline which is active against Chlamydia trachomatis and Ureaplasma urealyticum, the next most frequent cause of NGU (1). The Center for Disease Control has recommended that tetracyclines be included in treatment regimens for gonorrhea and that men with NGU and their sexual contacts be treated routinely with tetracyclines. Similar recommendations have been made for women with mucopurulent endocervicitis, the urethral syndrome (frequency and dysuria without significant bacteruria) and acute salpingitis. These recommendations were made because penicillin, the treatment often used for some of these conditions, is not effective therapy for chlamydial infections (8). While presumptive indicators for symptomatic patients may exist, accurate laboratory diagnosis is extremely important for two main reasons. First, to assure appropriate diagnosis and treatment for symptomatic patients and secondly, to prevent further transmission and complications in the large number of asymptomatic patients occuring in the population (particularly women) who serve as a reservoir.

In addition to genital infection, C. trachomatis is the cause of several ocular infections. Trachoma, a chronic conjunctivitis, is endemic in many developing nations (North

Africa, sub-Saharan Africa, the Middle East and Southeast Asia) and is the world's leading cause of preventable blindness. Active infection occurs mainly in children and the disease follows familial patterns. The natural course of uncomplicated trachoma appears to be relatively self-limiting in terms of active disease or inflammation. Unfortunately, in endemic areas reinfection is common and secondary bacterial infections interfere with the tendency to heal, thus prolonging the inflammatory process for years. Active disease does eventually disappear, usually by 10-15 years of age. The blinding complications of the disease take years to develop and blindness often occurs 25 or more years after active disease (3). Topical ocular chemotherapy with tetracycline ointment is effective in minimizing the severity of trachoma and therefore reduces the incidence of consequent blindness (1).

Adult inclusion conjunctivitis is almost invariably acquired by exposure to infective genital tract discharges. The disease is generally considered to be benign and self-limited. Systemic therapy with tetracyclines (rather than topical therapy) is used to treat patients and their sexual contacts because they both almost invariably have infections of the genital tract (1).

Both trachoma and inclusion conjunctivitis can be diagnosed by direct staining of conjunctival scrapings with Giemsa stain for inclusions; however, isolation in tissue

culture is more sensitive.

The entire spectrum of chlamydial infections has yet to be defined. The following diseases have been described in association with chlamydial infection: Reiter's syndrome, endocarditis, pericarditis, pharyngitis, and adult pneumonias, but the relation of these diseases to infection is still unclear (19).

The laboratory diagnosis of C. trachomatis infection involves the following procedures: 1)serology, in which anti-chlamydial antibodies are measured 2)isolation, in which the organism is cultured and 3)cytology, in which the typical intracytoplasmic inclusion or elementary body is demonstrated in stained specimens.

1)Serology

Serological tests for demonstrating anti-chlamydial antibodies include the complement fixation (CF) test and Wang's microimmunofluorescent (MIF) technique. The CF test has been used in the diagnosis of psittacosis and lymphogranuloma venereum. The MIF test, which uses a number of specific antigens, was first introduced for the serotyping of trachoma-inclusion conjunctivitis agents, and has been applied extensively in seroepidemiologic studies on both ocular and genital tract infections. It is more sensitive than the complement fixation test.

Serology is not very useful in diagnosing chlamydial infections. Seroconversion is difficult to demonstrate

because either the infection is low-grade in nature, the patient is seen relatively late in the course of the infection or the patient is suffering from reinfection. The high backround prevalence rates of anti-chlamydial antibody make serological tests of limited use for diagnosis. For instance, among sexually active young adults, the prevalence of seropositivity is aproximately 25% for males and 40%-70% for females (4). Thus, as a diagnostic test a single positive titer could only be used to determine previous exposure. IgM antibodies in the MIF test may give greater support for the diagnosis of an active or recently acquired infection, but only about 30% of people with active infection have these antibodies and most patients who have IgM antibodies do not have demonstrable chlamydial infection (4).

In systemic infections such as salpingitis, epididymitis and lymphogranuloma venereum, the larger antigen load appears to cause higher antibody titers and serodiagnosis may be helpful. The single condition where high serodiagnosis may be the method of choice is chlamydial pneumonia in infants where high levels of IgM antibody appear to be diagnostic. IgG antibodies are not useful because the infants often have high titers of maternally acquired antibodies (8).

2)Isolation

Because chlamydiae are obligate intracellular parasites

they cannot be grown on artificial media and tissue culture facilities are required. Chlamydia isolation can be performed on blood, sputum, throat washings, pleural fluid, vomitus, bubo pus (for diagnosis of lymphogranuloma venereum), fecal samples from birds (for isolation of C. psittaci), tissue biopsies, and conjunctival scrapings (for diagnosis of inclusion conjunctivitis and trachoma). In the case of genital infection, collection of appropriate epithelial cells is critical for chlamydial culture. Sampling of discharge is inadequate. Urethral specimens should be taken from several centimeters within the urethra. Cervical specimens should be collected from within the endocervical canal because chlamydial infections are restricted to columnar and squamocolumnar cells which are not found in the ectocervical (squamous) cells (4).

The specimens, after receipt in the laboratory, are homogenized and then inoculated into the cell monolayers by centrifugation. The cells are then incubated with cycloheximide (an anti-metabolite) in tissue culture medium for 48 to 72 hours and examined microscopically for detection of inclusions.

Inclusions may be demonstrated in infected tissue culture by staining with iodine, Giemsa stain or fluorescent antibody against Chlamydia. Iodine staining is simple and commonly used though the fluorescent antibody (FA) procedure, is more sensitive and can demonstrate inclusions

at less than 48 hours incubation (44). Negative cultures may undergo a blind passage at 4 days. This increases the likelihood of recovering Chlamydia by 15% to 35% (45). The introduction of microtiter plates has miniaturized tissue culture (TC) systems but is not as sensitive as the vial method that employs coverslips (44). The tissue culture technique is still the method of choice for diagnosing chlamydial infection in patients with virtually all of the chlamydial diseases. Its disadvantages are that tissue culture facilities are available in relatively few laboratories, it is expensive and 3 to 7 days are required to obtain results.

3)Cytologic

The classic procedure for demonstrating C. trachomatis in direct clinical specimens has been the Giemsa stain for inclusions. Compared to tissue culture, the Giemsa stain is a relatively insensitive diagnostic method (40). It is still used in staining conjunctival scrapings of patients with inclusion conjunctivitis and trachoma, however a trained microscopist who can recognize inclusions and distinguish them from artifacts or other structures is required and it takes approximately 30 minutes to adequately evaluate a single specimen.

FA staining of epithelial cell scrapings is far more sensitive than Giemsa staining although less sensitive than tissue culture isolation (40,41,42). The recent

availability of anti-chlamydial monoclonal antibodies has made it possible to detect elementary bodies in clinical specimens with greater accuracy and less backround fluorescence.

The MicroTrak™ Chlamydia trachomatis Direct Specimen Test (Syva Co., Palo Alto, CA) has been presented as a rapid alternative method of detecting C. trachomatis infection. The test uses fluorescein-conjugated monoclonal antibodies, reactive with all 15 known human serovars of C. trachomatis, to detect elementary bodies in smears (27, 28). As with tissue culture technique, proper specimen collection is crucial for demonstrating Chlamydia. By allowing diagnosis in settings where tissue culture is not available and by providing diagnosis within 30 minutes after the specimen is taken, direct specimen tests employing monoclonal antibodies to Chlamydia may be helpful in controling C. trachomatis infections, especially in high risk populations and in endemic trachoma control programs.

The purpose of this study was to compare the diagnostic capabilities of fluorescein-conjugated monoclonal antibodies reactive with Chlamydia trachomatis to:

I. conventional culture methods in cervical and/or endometrial specimens in 3 populations of women.

II. Giemsa staining in conjunctival smears from trachoma patients.

MATERIALS and METHODS

I. Identification of Chlamydia in cervical, endometrial and PAP smears.

A. Patient populations. Cervical specimens were received from 3 populations of females: 1)asymptomatic females in an adolescent clinic (AC) 2)asymptomatic females attending a planned parenthood clinic (PP) and 3)patients with acute salpingitis (AS). Endometrial specimens were collected from women with acute salpingitis attending the AS clinic. PAP smears were also collected from women attending the AC clinic. Women who had received antibiotics in the previous 2 weeks were excluded from the study.

B. Specimen collection. Duplicate endocervical specimens were collected from women using a Dacron swab for the MicroTrak Direct Specimen Test and a calcium alginate swab for culture. Sampling order was randomized. The exocervix was first cleaned with a cotton or Dacron swab to remove mucus and exudate. Another Dacron swab was inserted into the endocervical canal, rotated against the wall and removed, avoiding contact with any vaginal surface. The swab was rolled onto the MicroTrak slide, covering the 8mm well evenly with specimen. The slide was air dried, fixed by flooding with 100% acetone, and air dried. Fixed slides were stored at 4 C until staining. A calcium alginate swab was collected the same way and then immersed in transport medium (2.0 ml of Eagle's MEM in Earle's salts containing

10% fetal calf serum, 1% L-glutamine (200 nM solution), 10 µg per ml of gentamicin, 100 µg per ml of vancomycin, 10 units per ml of nystatin, and 3 µmoles/ml of glucose) for culture. Specimens were stored at 4 C for up to 72 hours before inoculation of cells.

Duplicate endometrial specimens were obtained from patients with acute salpingitis by passing a triple lumen catheter through the cervical os into the uterus. Sampling of the endometrium was done by brush biopsy. These slides were handled in the same manner as cervical slides. Brush biopsy samples of the endometrium obtained for culture were immersed in transport medium and stored up to 72 hours for culture isolation.

PAP smears, when taken, were obtained first and reflected a representative sampling of the endo-ectocervical epithelium. PAP smears were prepared by cervical sampling using both an Ayres spatula and an endocervical swab. A calcium alginate swab was inserted into the endocervical canal, rotated and then withdrawn without touching any vaginal surface. The swab was rolled onto a microscope slide. The Ayres spatula was used to make a 360° scrape of the endo-ectocervix and this sample was added to the sample from the endocervical swab. Smears were fixed for 10 minutes in cold acetone, air dried and immediately frozen at -70C until staining.

C. MicroTrak Procedure.

1. Cervical and endometrial specimens: The MicroTrak reagent contains fluorescein-conjugated monoclonal antibodies against the chlamydial major outer membrane protein and a counterstain (Evan's Blue) in a protein-stabilized buffer solution. The acetone fixed slides were incubated with 30 µl of the reconstituted FA reagent for 15 minutes at room temperature in a moist chamber. Slides were then rinsed in distilled water, allowed to air dry and cover-slips were applied using the MicroTrak mounting fluid (PBS glycerol with anti-photo bleacher). A Zeiss fluorescent microscope equipped with epi-illumination, a quartz halogen 12 V 100-W light source and filter system for fluorescein isothiocyanate examination was used. Patient and control slides were screened at 400X magnification and all positive slides were confirmed at 1000X magnification. Every field within the well was scanned. Slides were scored as positive only if 10 or more smooth, evenly fluorescing bright apple green discs consistent with chlamydial elementary bodies were seen. Negative slides contained fewer than 10 fluorescing chlamydial particles per well. All slides were graded as follows: 0=no particles seen, 1=<10 particles, 2=10-25 particles, 3=>25 particles seen on a slide. Thin acellular smears containing only squamous cells or polymorphonuclear leukocytes indicated that an inadequate specimen had been collected and the specimen pair

was excluded from analysis.

2. PAP smears: MicroTrak procedure was performed as described for cervical and endometrial specimens, except PAP smears were stained with 60 µl of the reconstituted FA reagent instead of 30 µl.

D. Cell culture method. Chlamydia cultures were carried out in cycloheximide-treated McCoy cells in 1-dram shell vials, using a modification of the procedure of Ripa and Mardh (30). McCoy cell monolayers seeded on coverslips with growth media were freshly prepared in our laboratory, incubated at 35 C in 5% CO_2 and used at 3 days. Monolayers were checked for confluency on an inverted microscope before inoculation and sterility cultures were found to be free from bacterial contamination. Cells were prepared for inoculation by aspirating growth medium from the vial and washing with 1 ml of PBS. Specimens were processed on the day of receipt and specimens which had been previously collected 72 hours or longer were considered too old to process. Specimens for which cytopathic effect (cpe) was observed (atypical cells observed in tissue culture layer due to yeast, bacterial contamination or otherwise unhealthy cells) were reported "cpe" if observed on first passage and if no chlamydial inclusions were detected. Specimen tubes containing swab and glass beads were vortexed for 2 minutes and 1 ml of isolation media was added to each specimen tube and mixed. Approximately 0.7 to 1.0 ml of this sample

mixture was inoculated into each of 3 vials and centrifuged onto the monolayers (2700 g for one hour) at room temperature. After centrifugation the medium was aspirated and isolation media containing 0.5 µg/ml cycloheximide was added to each vial. After incubation at 35 C in a CO_2 incubator for 3 days, 1 vial of each specimen was fixed with methanol and stained with iodine. Coverslips were removed from the vials and examined microscopically for intracytoplasmic inclusions of C. trachomatis. Four days after inoculation the 2 remaining vials were pooled for a second (blind) passage, done in the same manner. Cultures were graded as follows: 0=negative culture, 1=<25 inclusions, 2=25-100 inclusions, 3=>100 inclusions per coverslip.

E. Statistical comparisons. Sensitivity, specificity, and predictive values were determined from binary or "2 by 2" tables as shown in table 2. Our reference method, tissue culture (TC), was assumed to be 100% correct. Each cell represents the number of women who fall into one of four groups, depending upon whether or not she has a TC-positive or TC-negative result and a FA-positive or FA-negative result. Referring to table 2, the following calculations were determined :

		TC +	TC -	totals:
FA	+	TP	FP	TP+FP
	-	FN	TN	FN+TN
totals:		TP+FN	FP+TN	TP+FP+TN+FN

$$\text{sensitivity} = \frac{TP}{TP+FN} \times 100$$ (ability of test to identify people who are culture positive)

$$\text{specificity} = \frac{TN}{FP+TN} \times 100$$ (ability of test to identify people who are culture negative)

$$\text{predictive value (+)} = \frac{TP}{TP+FP} \times 100$$ (probability of being culture positive given a positive test)

$$\text{predictive value (-)} = \frac{TN}{FN+TN} \times 100$$ (probability of being culture negative given a negative test)

$$\text{prevalence} = \frac{TP+FN}{FP+TP+FN+TN} \times 100$$ (percentage of people who are culture positive at the time of the study)

true-positives= TP (specimens that were positive with both methods)

true-negatives= TN (specimens that were negative with both methods)

false-positives= FP (culture-negative specimens that test positive by FA)

false-negatives= FN (culture-positive specimens that test negative by FA)

TC= tissue culture

FA= fluorescent antibody test

Table 2. Use of the binary table to determine sensitivity, specificity, predictive values and prevalence (29).

True-positive (TP) specimens were those specimens that were positive with both the method being tested (FA) and the method used as the standard (TC). Similarly, true-negative (TN) specimens were negative with both methods. False-positive (FP) specimens were positive by FA but negative by TC and, therefore, were regarded as being truly negative. False-negative (FN) specimens were negative by FA but positive by TC and were regarded as being truly positive.

MATERIALS and METHODS

I. Identification of *Chlamydia* in trachoma specimens.

A. Patient population. In this study conjunctival scrapings were taken from children with active trachoma in a trachoma endemic area of southern Tunisia.

B. Specimen collection. Two sets of slides were taken from children examined during treatment trials or surveys. The conjunctival scrapings, obtained with a Kimura spatula from each eye, were spread thinly on double well glass slides (10mm diameter wells). The matched slides were allowed to air dry before being fixed with cold acetone for FA staining or with absolute methanol for Giemsa staining. The slides were transported to San Francisco and stored at -70 C until staining.

C. MicroTrak Procedure. The acetone fixed slides were incubated with 40 µl of the reconstituted FA reagent per well (80 µl per slide) for 15 minutes at room temperature in a moist chamber. Slides were rinsed in distilled water, allowed to air dry and coverslips applied using phosphate buffered saline with glycerol. Slides were scanned at 400X and confirmed at 1000X using a Zeiss epifluorescent microscope. Every field was scanned carefully and approximately 10-15 minutes was required to read each slide. Slides were judged positive if at least 10 elementary bodies were seen in either well of the slide. The scoring of slides for elementary bodies was as follows: Light=1+ (<5

org/hpf), Moderate=2+ (5-20 org/hpf) and Heavy=3+ (>20 org/hpf). The presence of intact inclusions in cells was also noted.

D. Giemsa Staining Procedure. The methanol fixed slides were stained for 1 hour with Giemsa stain buffered at pH 6.8, rinsed briefly in 95% ethanol to remove excess dye, dried and then examined for the presence of typical basophilic intracytoplasmic inclusion bodies (31). The entire double well slide was examined microscopically for at least 20-30 minutes.

E. Statistical Comparisons. Sensitivity, specificity and predictive values were calculated using standard techniques previously described in table 2, page 21, however, Giemsa stain was considered to be the gold standard.

RESULTS

I. Cervical

Of 1230 specimens collected for this study, 262 matched results were unavailable for comparison. Of these 262 specimens, 150 were inadequate FA smears, 64 were inadequate for culture (too old to process or inconclusive result due to cytopathic effect) and the remaining unacceptable due to mislabeling or missing paired specimens. The results of the remaining 968 paired specimens (FA & TC) are shown in Table 3. The prevalence of chlamydiae by culture was 13% (126/968). Compared to culture, FA sensitivity was 70% (88/126); and specificity was 94% (795/842). There was 91% agreement (883/968). Predictive value of a positive FA test was 65% (88/135) and negative FA 95% (795/833). We reexamined 38 smears where paired results were discrepant and the result changed in only 5 due to reread (2 from + to- and 3 from - to +). The comparison of FA to TC by clinic is shown in Table 4. In different clinics the sensitivity ranged from 63% to 76%; specificity from 92% to 97% and the predictive value of a positive test from 55% to 77%. While the differences are not statistically significant the variation suggests that differences in specimen handling did exist.

A comparison of the degree of positivity between FA and TC results is shown in Table 5. A total of 45 MicroTrak specimens were given a score of 1 (<10 elementary bodies

were seen per slide). Forty one of these 45 paired isolation attempts were negative by culture and 4 were positive by culture. If we had used a cutoff value of 1 elementary body per slide for a positive MicroTrak specimen, an additional 41 false- positive FA slides would have resulted. The overall sensitivity of the test would have changed from 70% to 71%, but specificity would have dropped from 94% to 89% and the predictive value of a positive test would have decreased from 64% to 51%. Thus the recommended cutoff value of 10 elementary bodies or more as the criterion for a positive MicroTrak Test was supported by our data.

II. PAP

Of 140 PAP smears collected, 24 matched results were unavailable for comparison. Of these 24 specimens, 7 cultures were unreadable because of yeast contamination, 4 cultures were too old to process, 8 cultures were lost, and 5 PAP smears were unreadable because they contained no cells. The results of the remaining 116 paired specimens (FA and TC) are shown in table 6. The prevalence of chlamydiae by culture was 21% (24/116). Compared to culture, FA sensitivity was 63% (15/24), and specificity was 99% (91/92). There was a 91% agreement (106/116). Predictive value of a positive test was 94% (15/16) and negative FA 91% (91/100).

III. Endometrial

Of 90 endometrial specimens collected; 18 matched results were unavailable for comparison. Of these 18 specimens, 14 were insufficient smears and 4 specimens were missing matched pairs. The results of the remaining 72 paired specimens (FA and TC) are shown in table 7. The prevalence of chlamydiae by culture was 17% (12/72). Compared to culture, FA sensitivity was 67% (8/12); and specificity was 97% (58/60). There was a 92% agreement (66/72). Predictive value of a positive FA test was 80% (8/10) and negative FA 94% (58/62).

IV. Trachoma

Of 168 paired trachoma slides evaluated, 13 were either Giemsa or FA insufficient smears. The results of the remaining 155 paired slides are shown in table 8. The prevalence of chlamydiae by Giemsa stain was 23% (36/155). Compared to Giemsa stain, FA sensitivity was 61% (22/36) and specificity was 95% (113/119). There was 87% agreement (135/155). Predictive value of a positive FA test was 79% (22/28) and negative FA 89% (113/127). If we had used a cutoff value of 1 elementary body per well for a positive FA slide, an additional 11 false positive and 6 true positive FA slides would have resulted and the overall sensitivity of the test would have changed from 61% to 78%, but specificity would have dropped from 95% to 86% and the predictive value of a positive test would have decreased from 79% to 62%.

Cervical and endometrial smears averaged 3-5 minutes to read, depending on the quality of the smear. PAP smears required about 10 minutes per slide to read due to greater surface area. The presence of artifacts on the slide increased the time required for interpretation. Positive slides were usually evaluated within 2 minutes. Only extracellular elementary bodies, visualized microscopically as discrete, evenly fluorescent bright apple green discs ~300 nm in diameter or occasionally larger reticulate bodies were observed. No intact chlamydial inclusions were seen in these smears.

Trachoma FA stained smears required about 10-15 minutes per slide to read (due to greater surface area and more artifacts). Extracellular elementary bodies as well as intact inclusions (in 10 of the 155 slides) were seen in these smears.

DISCUSSION

I.Comparison of monoclonal antibody staining and culture in diagnosing cervical chlamydial infection.

The FA system was less sensitive than culture (Tables 3 and 4). The MicroTrak Direct Specimen Test detected 70% of culture-positive endocervical chlamydial infections in a largely asymptomatic population of women. The large number (12%) of inadequate MicroTrak specimens received indicates a need for clinician training in specimen collection. False-negative results (FA-,TC+) occurred mainly in patients whose infections were characterized by low number of organisms often requiring a second TC passage for detection. Lowering the cutoff value to 1 elementary body to try and identify these low grade positives did not improve sensitivity but did compromise the specificity of the test. Our results support a cutoff value of 10 elementary bodies or more as criterion for a positive MicroTrak test.

Some false positive results (FA+, TC-), occur because TC is less than 100% sensitive. Other false positive results may in fact have been obtained for women with true chlamydial infections, since dead or otherwise noninfectious organisms can be stained by antibody in the FA test. Unfortunately, it is not possible to identify which of this group is a true false positive. There are other reasons for false-positive results. Since different swabs were used to obtain specimens for each culture and smear, variable

sampling of the cervix may have accounted for some of the discrepant results. Twelve percent of our pairs were excluded from analysis because the smear had inadequate cells. Although slides underwent microscopic evaluation, specimens submitted for culture could not be evaluated for adequacy. If the inadequate specimens were randomly distributed between the two tests we would have missed 20 positive cultures that would have been detected had an adequate specimen been taken. If that conjecture is valid there would be a slight improvement in performance of the test (sensitivity 74%, specificity 97%, and the predictive value of a positive test 80%). It is likely that many highly positive FA, culture negative specimens (EB score >25, Table 5) represent inadequate or improperly handled culture specimens.

Another potential cause of false-positive FA is cross-reacting antigen in the specimen. The assumption that because the reagent is a monoclonal antibody it will only stain C. trachomatis is wrong. We have seen 3 different bacteria fluoresce in this test (Neisseria species, and two morphologically different cocci). The morphology is more likely to be confused with reticulate bodies than elementary bodies- but that statement is based on slides where we could make the differentiation- not on slides we misidentified and thus created false-positives. There is a subjective element associated with reading the direct immunofluorescence test

and a careful program of training and standardization is required for proper interpretation of this test.

Our results suggest that the MicroTrak Direct Specimen Test would be a useful test for diagnosing chlamydial infection in high prevalence populations. For low risk groups however, our projections suggest that the predictive value of a positive test would be low. For example, projection of our results (sensitivity 70% and specificity 94%) onto a population of 1000 women with a 5% prevalence of chlamydial infection (Table 9) would result in correct identification of 35 of 50 infected women, missing 15. Of greater concern is the fact that 57 false positives would result from this test; thus the predictive value of a positive MicroTrak Test would only be 35 of 92 or 38%. A projection of our results into a high risk population with a 25% prevalence of chlamydial infection would result in a predictive value of a positive MicroTrak Test of 79% and a predictive value of a negative MicroTrak test of only 90%. Thus there is no certainty that chlamydial infection could be ruled out by a negative MicroTrak Test in such a high risk population.

Previous studies have reported a much better performance of the MicroTrak Direct Specimen Test when compared with tissue culture (28,32-34). Tam et al (28) reported a sensitivity of 93%, a specificity of 96% and a predictive value for a positive test of 87%, when FA was

compared with TC in McCoy cells (96 well microtiter plates, blind passage and iodine staining). This study was performed in a population with a high prevalence of chlamydiae (~22%) who presented with clinical syndromes known to be associated with chlamydial infection: men and women with overt urethritis or mucopurulent cervicitis, respectively. Such patients typically have higher inclusion counts than unselected patients. The improved performance of the MicroTrak Direct Specimen Test probably resulted in part from the patient selection and in part from the culture technique employed in this study: 96-well microtiter plates are less sensitive than vials for detecting inclusions in tissue culture.

Thomas et al (32) also reported excellent agreement (94%-99%) and sensitivity (91%-100%) in their comparison study of FA stain with single pass culture in a high risk population. This patient population consisted of men with NGU, men with gonorrhoea, female contacts of men with NGU and babies with conjunctivitis. Stamm et al (33) have reported a sensitivity of 92% in 576 men, most of whom had symptoms and signs of urethritis, and 89% sensitivity for 595 high risk women attending the same sexually transmitted disease clinic. Uyeda et al reported a sensitivity of 96%, a specificity of 99% and a predictive value for a positive test of 92% (34). This study was of 401 asymptomatic females who were either pre- or post-abortal

patients. Prevalence of chlamydial infection in this population was ~7%. The improved performance of the MicroTrak Direct Specimen Test in this case may have been due to the less sensitive culture technique employed. McCoy cells used were not prepared fresh as in our study. Their comparison of the FA was with first passage tissue culture (an additional 15-30% positives may have been picked up if a second passage had been done).

There may be other reasons for a better performance of the MicroTrak Direct Specimen Test. In two of the above mentioned studies (Stamm et al and Uyeda et al), a cutoff value of 2 or more elementary bodies was used as the criterion for a positive test. This may have increased the sensitivity of FA by increasing the number of false positives. Specificity, however, did not seem to be compromised in either of these studies.

A comparison of FA staining in PAP and cervical smears with culture (tables 6&4) showed no significant difference in the performance of the MicroTrak Test for either of these specimens. There were 21% culture positives in the PAP smear comparison and 13% culture positives in the cervical comparison. The greater number of culture positives in the former (reflected in the greater positive predictive value for the FA stained PAP smear) was due to the selection process for adolescents chosen for PAP smears. For cervical smears, where the percentage of culture positives was lower,

the predictive value for a positive cervical FA test was also low.

The detection of chlamydiae in PAP smears has not been standardized. In our study the PAP specimen was smeared over most of a microscope slide and thus required more time for evaluation. Specimens which were confined to 8 mm wells with defined opaque borders (ie. cervical and endometrial slides) were more concentrated and easier to read. Also, less reagent and reading time was required for these slides. While cellular changes seen on PAP smears (though not specific for chlamydial infection), could be used to screen for women who should be tested for chlamydial infection (35), FA stained smears would not allow for observation of inflammatory patterns and in addition, evaluation would be tedious and time consuming.

II. Comparison of monoclonal antibody staining and culture in detection of chlamydial infection in endometrial smears.

A comparison of FA staining in endometrial and cervical smears (tables 7 and 4) shows cervical specimens to be a more sensitive indicator of chlamydial infection in this population of women. Of 12 matched specimens that were cervical culture positive, 83% (10/12) were endometrial culture positive and only 50% (6/12) were endometrial FA positive. Theculture method used in this study, therefore, seems to be more sensitive than FA in detecting chlamydial

infection in endometrial specimens.

The prevalence of chlamydiae by culture in this population was between 17%-20%. This finding concurs with other findings. When culture results from four studies from the United States on acute salpingitis were added together, 15.4% (27/175) patients with acute salpingitis were culture positive for C. trachomatis from the lower urogenital tract (36).

III. Comparison of monoclonal antibody and Giemsa staining in detection of chlamydial infection in trachoma.

The FA procedure was not as effective as Giemsa stain for identifying chlamydial infection in conjunctival smears from children with endemic trachoma (table 8). More positives were detected by FA if the presence of at least 1 elementary body was considered to be diagnostic, but this sacrificed the specificity and positive predictive value of the FA test (page 29).

The sensitivity of FA for diagnosing trachoma in conjunctival scrapings in our study was considerably less than that reported in other studies of genital and ocular infections. The performance of the FA test in genital specimens has already been discussed (pages 29-34). Bell et al (37) studied the FA performance in diagnosing 39 infants with neonatal conjunctivitis. Complete concordance between smears and culture was found for 55 specimens from inflamed

conjunctiva of 23 infants with unilateral and 16 with bilateral conjunctivitis (FA stain, 37 C, 30 min., + criterion >5 EBs). Taylor et al (38) used a monkey model of chlamydial infection to compare FA, Giemsa stain, and TC in 110 paired specimens (FA stain, RT, 15 min., + criterion >3 EBs). They found Giemsa cytology to be very insensitive (sensitivity, 30%) though highly specific (100%) and FA cytology to be highly sensitive (94%) but specificity low (70%) when compared to TC (2nd pass, 96 well microtiter plates, FA staining of inclusions). Ugland et al (39) found disparate FA results for 9 specimens (5 FP & 4 FN) from 32 adults: 10 normal volunteers, 13 patients with adenoviral conjunctivitis, and 9 patients with chlamydial conjunctivitis.

There could be several reasons for this disparity among studies including specimen collection, variability in microscopic interpretation, and low sensitivity of Giemsa stain. The method for handling eye smears in this study differed from the methods recommended for the FA test in several ways (table 10). Specimen scrapings were less concentrated and spread thinly (1 cell thick) over slide wells (unlike FA recommendations for genital specimens in which swabs are spread thickly and evenly over 8 mm wells). Scraping the conjunctiva favors the preservation of intact inclusions, whereas swabbing may disrupt intracytoplasmic inclusions and provide more elementary bodies for staining.

Since our diagnosis is dependent on the detection of free elementary bodies, swabbing the conjunctiva may be the optimum method for obtaining specimens. There also seemed to be more artifacts and nonspecific staining in trachomatous conjunctival smears, making microscopic interpretation rather difficult and uncertain. As discussed previously, the FA also stains other organisms which could have been mistaken for elementary bodies and this would create additional false positives. Neisseria species, when recognized, were seen fluorescing in both genital and ocular specimens. Criteria for identifying intact inclusions in cells have not been established for the FA procedure in conjunctival specimens. Of the 10 slides where inclusions were noted, all 10 contained free elementary bodies and all but 1 were Giemsa positive. Further evaluation of the FA procedure will be necessary to determine the optimum conditions for collecting and staining eye smears and to establish the criteria for the identification of chlamydial inclusions.

Finally, Giemsa stain, though highly specific, is less sensitive than tissue culture in detecting chlamydial infection in trachoma (40) and many FA positive, Giemsa negative slides may be representative of true infections. Previous studies comparing polyclonal FA with Giemsa stain have shown that positive results were obtained more often with polyclonal FA (based on presence of inclusions) than

with Giemsa stain (41,42). Results with the Giemsa stain correlated well with active disease whereas polyclonal FA had a relatively poor correlation with clinical findings. In our present study utilizing monoclonal FA, we also noted a higher rate of FA positivity (based on presence of EBs) in absence of Giemsa staining inclusions. These observations may indicate that the FA technique is truly more sensitive and less specific than Giemsa or alternatively, that only the most actively infected individuals were detected by the Giemsa method, while the more sensitive FA technique detected clinically inapparent infections as well as clinically active infections. Unfortunately, clinical data was not available to make a comparison between the results obtained by microscopic examination of conjunctival scrapings with clinical diagnosis. More inclusion positive slides were detected by Giemsa (36 slides) than by FA (10 slides). One explanation is that the composition of the MicroTrak Direct reagent is more conducive to staining free elementary bodies than intracytoplasmic inclusions. Also, the lack of guidelines for identifying FA stained inclusions, their resemblance to artifacts and their yellowish color in cells posed problems in identification and may have resulted in a more conservative estimate of inclusion presence.

Though laboratory tests are not essential to trachoma control programs, they may be used for a number of reasons:

to support the clinical diagnosis of individual cases, to monitor the effect of therapy, to measure prevalence in field studies in endemic areas, and to detect serotype shifts in a given population that might indicate the influx of agent from outside the community or possible transmission of non-ocular serotypes (43). A variety of laboratory methods may be used for the diagnosis of trachoma. The choice of method depends on various factors such as sensitivity, cost and availability of the test procedure, as well as its ability to deal with the required test volume and the skill level required.

The measurement of type-specific antibodies in the microimmunofluorescence (MIF) test applied to serum or tears offers the most sensitive index of exposure to trachomatous infection. The simplicity and availability of serial specimens of tears in many areas in which the collection of serum is difficult, makes the application of the MIF test to tears a convenient method. However, serum antibodies may persist after active infection, whereas the presence of antibodies in tears appears to more closely related to active disease (31).

In field studies in which high sensitivity is desirable for either small or large scale work, isolation in cell culture has great advantages. Although it requires more complex facilities than inclusion finding methods in conjunctival smears, it requires less technologist time and

is more sensitive. Furthermore, it yields isolates for serotyping and the study of other biological characteristics. Its successful application depends on the maintenance of suitable conditions of storage and tansportation of specimens from the field to the laboratory (31). In field studies where facilities to isolate chlamydiae are not available, methods of demonstrating inclusions in conjunctival scrapings may be of particular value, if suitably trained microscopists are available.

Giemsa staining is the preferred method for demonstrating inclusions in scrapings from patients with trachoma because it is specific and provides other information on cytology and bacterial infection. It is also useful in providing permanent preparations that can be sent to specialized laboratories for confirmation of inclusions and is the most readibly available and reliable method. Staining with fluorescein-labeled polyclonal antibodies against C. trachomatis was more complex, costly and more time consuming than Giemsa staining and is no longer used (31). FA staining using monoclonal antibodies requires less time and the microscopy is less fatiguing and more rapid than with Giemsa staining. However, the successful use of FA methods, like cell culture, depends on the availability of adequate facilities for storage and transportation of refrigerated specimens. FA slides are best examined within 24-48 hours of staining and require an epifluorescent

microscope with the proper filter system for observation. Also, FA slides cannot be stored indefinitely and cytology cannot be evaluated. Fluorescein-labeled monoclonal antibodies may offer a rapid alternative to Giemsa stain in diagnosing C. trachomatis infection in field studies of trachoma but its efficacy in diagnosing ocular chlamydial infection still remains uncertain.

CONCLUSION

In summary, the FA procedure has a performance profile which could make it a useful tool in screening for genital infection in high risk populations (particularly when tissue culture is not available) but it is less well suited to screening low risk populations where false positives will become more important. For situations where facilities to isolate chlamydiae are not available, the examination of conjunctival smears by the FA procedure offers an alternative to isolation and may be useful for the rapid diagnosis of individual cases and for epidemiological and therapeutic studies.

Table 3

Sensitivity, Specificity, Predictive values and Prevalence for the Comparison of FA and TC in the diagnosis of cervical chlamydial infection, n=968*

		TC, 1st pass +	TC, 1st pass -	TC, 2nd pass +	TC, 2nd pass -
FA	+	55	80	88	47
FA	-	16	817	38	795

	TC, 1st pass	TC, 2nd pass
sensitivity	77%	70%
specificity	91%	94%
+ predictive value	41%	65%
- predictive value	98%	95%
prevalence	7.3%	13%

*Of 1230 specimens received, 262 were unsuitable for comparison
150 (12%) FA smears were inadequate
55(4%) TC tests were unsatisfactory

Table 4

Sensitivity, Specificity, Predictive values and Prevalence for the Comparison of FA and TC (2nd Pass) in the diagnosis of cervical chlamydial infection by clinic

	ADOLESCENT CLINIC n=438	PLANNED PARENTHOOD n=426	SALPINGITIS CLINIC n=104
	TC + / TC −	TC + / TC −	TC + / TC −
FA +	37 / 11	35 / 29	16 / 7
FA −	22 / 368	11 / 351	5 / 76
sensitivity	63%	76%	76%
specificity	97%	92%	92%
+ predictive value	77%	55%	70%
− predictive value	94%	97%	94%
prevalence	13%	11%	20%

Table 5

Degree of Positivity of FA with TC in cervical specimens

		TC RESULT	
EB Score*	(n)	1st Pass +	POSITIVE
0	(788)	2%	4%
1-9	(45)	4%	9%
10-25	(54)	22%	54%
>25	(81)	53%	73%

EB Score*
0 organisms= negative, score 0
1-9 organisms= negative, score 1
10-25 organisms= positive, score 2
>25 organisms= positive, score 3

Table 6

Sensitivity, Specificity, Predictive values and Prevalence for the Comparison of FA stained PAP smears and TC in the detection of chlamydial infection, n=116*

		TC, 1st pass +	TC, 1st pass -
FA	+	11	5
	-	2	98

		TC, 2nd pass +	TC, 2nd pass -
FA	+	15	1
	-	9	91

sensitivity	85%	63%
specificity	95%	99%
+ predictive value	69%	94%
- predictive value	98%	91%
prevalence	11%	21%

*Of 140 specimens received, 24 were unsuitable for comparison:
5 (4) FA smears were inadequate
11 (8%) TC test were unsatisfactory
8 were missing matched pairs

Table 7

Sensitivity, Specificity, Predictive Values and Prevalence for the Comparison of FA and TC in the diagnosis of chlamydial infection in endometrial specimens, n=72*

		TC, 1st pass +	TC, 1st pass -	TC, 2nd pass +	TC, 2nd pass -
FA	+	4	6	8	2
FA	-	1	61	4	58

	TC, 1st pass	TC, 2nd pass
sensitivity	80%	67%
specificity	91%	97%
+ predictive value	40%	80%
-predictive value	98%	94%
prevalence	7%	17%

*Of 90 specimens received, 18 were unsuitable for comparison:
14 (15%) FA smears were inadequate
4 (4%) were missing matched pairs

Table 8

Sensitivity, Specificity, Predictive Values and Prevalence for the Comparison of FA and Giemsa stain in the diagnosis of trachoma, n=155*

		Giemsa stain +	Giemsa stain -
FA	+	22	6
	-	14	113

sensitivity	61%
specificity	95%
+ predictive value	79%
- predictive value	89%
prevalence	23%

*Of 168 specimens received, 13 (8%) were either Giemsa or FA insufficient smears

Table 9

Projection of our results for the comparison of FA and TC in cervical specimens (sensitivity 70%, specificity 94%) onto populations of 1000 women with low and high prevalence of chlamydial infections.

			+PREDICTIVE VALUE	-PREDICTIVE VALUE
1% PREVALENCE				
		TC +		
FA	+	7	10%	99.7%
	-	3		
		TC -		
FA	+	60		
	-	930		
5% PREVALENCE				
		TC +		
FA	+	35	38%	98%
	-	15		
		TC -		
FA	+	57		
	-	893		
20% PREVALENCE				
		TC +		
FA	+	140	74%	93%
	-	60		
		TC -		
FA	+	48		
	-	752		
25% PREVALENCE				
		TC +		
FA	+	175	79%	90%
	-	75		
		TC -		
FA	+	45		
	-	705		

Table 10

Summary of differences in FA methodology

	FA for detection of trachoma	FA recommendations
Specimens	conjunctiva	urethra cervix rectal
Instrument	spatula	swab
Slide	clear, double wells	clear 8mm wells opaque surrounding
Thickness	spread thinly	clumped in circle
Fixation	cold acetone	acetone or 95% ethanol, room temperature
Staining	15 minutes room temperature direct FA	15 minutes room temperature direct FA
Examination time	10-15 minutes	3-5 minutes
Criteria for diagnosis	elementary bodies or intact inclusions	elementary bodies

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