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ISOLATION OF CHLAMYDIA TRACHOMATIS

by

Sandra G. Norstedt

Bachelor of Science, University of North Dakota, 1978

A Thesis

Submitted to the Graduate Faculty

of the

University of North Dakota

in partial fulfillment of the requirements

for the degree of

Master of Science

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This thesis submitted by Sandra G. Norstedt in partial fulfillment of the requirements for the Degree of Master of Science from the University of North Dakota is hereby approved by the Faculty Advisory Committee under whom the work has been done.

James J. Feltner
(Chairperson)

James R. Waller

Gordon M. Fiebig

Robert Mordlie

This thesis meets the standards for appearance and conforms to the style and format requirements of the Graduate School of the University of North Dakota, and is hereby approved.

A. William Johnson
Dean of the Graduate School

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TABLE OF CONTENTS

LIST OF ILLUSTRATIONS	vi
LIST OF TABLES.	vii
ACKNOWLEDGEMENTS.	viii
ABSTRACT.	ix
INTRODUCTION.	1
Growth Cycle	2
Chlamydial Infections.	3
Diagnosis of Chlamydial Infections	6
MATERIALS AND METHODS	12
Tissue Cultures.	12
<u>Chlamydia trachomatis</u> Stock Cultures	13
Growth Medium.	13
Isolation of <u>C. trachomatis</u> in McCoy Cell Monolayer Cultures	14
McCoy Cell Monolayer Cultures	14
Inoculation and Centrifugation of Cell Cultures	14
Detection of <u>C. trachomatis</u> by Iodine Staining.	15
McCoy vs. L-929 Cells for Isolation of <u>C. trachomatis</u>	15
Isolation and Enumeration of <u>C. trachomatis</u> in McCoy Cell Suspension Cultures.	16
Evaluation of Temperature and Centrifugal Force Used for <u>C. trachomatis</u> Isolation	16
Effect of Inoculum-Suspending Medium on the Recovery of <u>C. trachomatis</u>	17
Storage at 4 ^o C.	18
Storage at -70 ^o C.	18
Comparison of Techniques of the Detection of <u>C. trachomatis</u> from Clinical Specimens.	19
Collection and Transport of Specimens	19
Isolation Procedure	19
Immunoperoxidase Assay.	20
Immunofluorescence Assay.	21
Quantitation of Results	22
Quality of Staining Techniques.	22
RESULTS	24
Comparison of McCoy and L-929 Cells for Isolation of <u>C.</u> <u>trachomatis</u>	24
Recovery of <u>C. trachomatis</u> from McCoy Cell monolayer and Suspension Cultures.	24

Effect of Centrifugal Force and Temperature on the Recovery of <u>C. trachomatis</u>	26
Evaluation of Inoculum-Suspending Transport Medium Used for <u>C. trachomatis</u>	28
Effect of Inoculum-Suspending Medium on the Survival of <u>C. trachomatis</u> Stored at 4°C.	28
Effect of Inoculum-Suspending Medium of the Recovery of <u>C. trachomatis</u>	28
Effect of Inoculum-Suspending Medium on the Recovery of <u>C. trachomatis</u> Stored at -70°C.	32
Comparison of Staining Techniques for the Detection of <u>C. trachomatis</u>	35
DISCUSSION.	44
SUMMARY	58
APPENDICES.	61
Appendix A. Key to Abbreviations.	62
Appendix B. Chlamydial Media and Stains	64
LITERATURE CITED.	65

LIST OF ILLUSTRATIONS

Figure	Page
1. Effect of Inoculum-Suspending Medium on the Survival of <u>C. trachomatis</u> stored at 4°C.	30
2. A. Iodine-staining of Chlamydial Inclusions in McCoy Cell Monolayer Cultures	39
B. Iodine-staining of Chlamydial Inclusions in McCoy Cell Monolayer Cultures	39
3. A. Immunoperoxidase (PAP) Staining of Chlamydial Inclusions in McCoy Cell Monolayer Cultures	41
B. Nonspecific Staining of McCoy Cell Monolayers With the PAP Assay.	41
4. A. Immunofluorescent (IFA) Staining of Chlamydial Inclusions in McCoy Cell Monolayer Cultures.	43
B. Nonspecific Staining of McCoy Cell Monolayers with the IFA Assay.	43

LIST OF TABLES

Table	Page
1. Relationship between chlamydial serotypes and human disease.	4
2. Recovery of <u>Chlamydia trachomatis</u> from McCoy cell monolayer and suspension cultures.	25
3. Effect of centrifugal force and temperature on the recovery of <u>C. trachomatis</u> from McCoy cell cultures.	27
4. Effect of inoculum-suspending medium on the recovery of <u>C. trachomatis</u> from McCoy cell monolayer cultures.	31
5. Effect of inoculum-suspending medium on the recovery of <u>C. trachomatis</u> after one freeze-thaw cycle at -70°C	33
6. Effect of storage at -70°C on the recovery of <u>C. trachomatis</u> in 2SP medium.	34
7. Comparison of <u>Chlamydia</u> -positive specimens detected by iodine staining and immunoperoxidase assay	36

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ABSTRACT

Chlamydia trachomatis is an obligate intracellular parasite responsible for a number of human infections. The growing prevalence of chlamydial infections has increased the demand for diagnostic testing for this organism. This study was performed to determine an effective and practical method for the diagnostic isolation of C. trachomatis in this laboratory.

McCoy and L-929 cell monolayers were compared for their susceptibility to C. trachomatis infection. In a preliminary study, the number of chlamydial inclusions counted was five-fold higher in McCoy cells than L-929 cells.

McCoy cell suspension cultures were tested as a means of simplifying tissue culture isolation of C. trachomatis by eliminating the need for pre-formed confluent cell monolayers. The recovery of C. trachomatis in suspended cells was comparable to the isolation rates in monolayer cultures in three of five trials, but the effectiveness of the suspension cultures was severely decreased by the presence of bacterial contamination and toxic products.

Recovery rates of C. trachomatis in McCoy cell monolayer cultures were compared with centrifugation performed for one hour at 500 x g at 36°C, and 900 x g at room temperature. Although the number of chlamydial inclusions enumerated after centrifugation at 500 x g at 36°C was almost double the number recovered with centrifugal forces at 900 x g at room temperature, at 36°C the temperature within the centrifuge could not be controlled. For subsequent studies,

centrifugation of the chlamydial inoculum was carried out for one hour at 900 x g at room temperature.

The following media were evaluated to find the optimal conditions for transport, storage, and inoculation of McCoy cell monolayer cultures: sucrose-phosphate medium (2SP), sucrose-phosphate-glutamate medium (SPG), Hanks' balanced salt solution, T-soy broth, 2SP containing 10% dimethyl sulfoxide, and 2SP containing 10% glycerol. 2SP medium was determined to be superior to the other media tested for storage of C. trachomatis, and as an inoculum-suspending medium. The recovery rates of C. trachomatis stored in 2SP medium decreased by approximately 20% per day at 4°C, and 50% after one freeze-thaw cycle at -70°C. No significant loss of viability of the organism was detected after prolonged storage at -70°C in 2SP medium.

Methods for the detection of chlamydial inclusions in McCoy cell monolayer cultures were compared to evaluate the effectiveness of iodine staining, immunoperoxidase (PAP) and immunofluorescence (IFA) assays. From 139 clinical specimens tested in parallel, iodine staining detected more Chlamydia-positive samples (9%) than the PAP assay (5%). Ten positive samples were frozen and tested a second time with iodine stain and the IFA assay. Only eight samples remained positive for chlamydiae with iodine staining and seven of the eight specimens were positive with the IFA assay. Iodine staining of cover-slip cultures was less expensive, less time-consuming, and easier to interpret than either the PAP or IFA assay.

Combining the use of McCoy cell monolayer cultures with iodine staining proved to be the most effective and practical method for isolation of C. trachomatis.

INTRODUCTION

Chlamydiae are obligate, intracellular parasites responsible for a wide variety of human and animal infections (1,2). They are common pathogens of many mammals and birds. Human diseases that are attributed to chlamydial agents include psittacosis, trachoma, lymphogranuloma venereum, inclusion conjunctivitis, and infections of the genital tract (3).

Chlamydiae were first seen in the early part of this century as intracytoplasmic inclusions in conjunctival scrapings from patients with trachoma. Originally, they were believed to be protozoa, but after the isolation of the psittacosis agent by Bedson in 1930, chlamydial agents were called viruses because they developed only in the cytoplasm of eucaryotic cells (2,4).

The eventual isolation and characterization of additional chlamydial agents led to the realization that chlamydiae were a unique type of infectious entity that shares many characteristics with bacteria (1). These characteristics include the presence of (i) DNA and RNA, (ii) a cell wall, (iii) enzyme systems, (iv) sensitivity to antibiotics, and (v) reproduction by a mechanism of binary fission (5). A pathway for the generation of ATP has not been detected for chlamydiae, so they are dependent upon ATP transported from the host cell cytoplasm (5,6).

Chlamydiae have been known by a number of different names over the years, and members of this group of organisms have been commonly classified with the rickettsiae (7). However, chlamydiae develop within the host cell by a cycle different from all other microorganisms, and this distinctive growth cycle has earned chlamydiae their own order, the Chlamydiales (1,8).

There is one genus and two species, Chlamydia psittaci and Chlamydia trachomatis.

Growth Cycle

The growth cycle of chlamydiae involves both an extracellular and intracellular phase. The extracellular phase is characterized by an infectious particle, or elementary body (EB). The elementary body is about 300 nm in diameter, metabolically inactive, and has a rigid, resistant cell wall similar to Gram-negative bacteria (5,9).

The elementary body interacts with the host cell surface and is taken up by a phagocytic process (10,11). The initial stages of attachment require an electrostatic interaction between the host cell and the parasite (12,13). Ingestion of chlamydiae is dependent upon binding of specific sites on the EB to complementary receptors on the host cell surface which induces phagocytosis (14,15). The induction of phagocytosis has been termed "parasite-specified" since "nonprofessional" phagocytic cells preferentially ingest chlamydial EB (15).

Chlamydiae remain in a phagosome throughout their growth cycle, but phagolysosomal fusion is inhibited by the organism (16). Host cell replication is not impaired unless chlamydiae are at a high multiplicity of infection (2).

Approximately twelve hours after entry into the cell, the EB undergoes reorganization to form the initial, or reticulate body (RB) (9). The RB is a strictly intracellular form of the organism; it is metabolically active, and has a fragile outer membrane (2,17).

The RB multiplies for several hours by binary fission until an inclusion which contains mostly reticulate bodies forms in the cytoplasm

of the cell. The reticulate bodies become reorganized to elementary bodies, and after 48 to 72 hours, the host cell bursts, releasing the infectious particles (9).

Chlamydial Infections

Chlamydia psittaci has the broadest range of the two chlamydial species, infecting primarily birds and mammals (1). Humans exposed to diseased birds can develop a pneumonia called psittacosis (3). Chlamydia trachomatis is a specifically human pathogen with 15 recognized serotypes (18). The relationship between chlamydial strains and human disease is shown in Table 1.

Chlamydia trachomatis serotypes L-1, L-2 and L-3 cause lymphogranuloma venereum (LGV). LGV is a venereal disease, characterized by inguinal lymphadenopathy, and is most prevalent in warm tropical climates (3). LGV strains are more invasive than the other C. trachomatis serotypes, infecting lymphoid tissue (serotypes A-K characteristically infect mucous membranes) (18).

C. trachomatis serotypes A, B, Ba, and C cause trachoma, the world's leading preventable cause of blindness (3,18). Trachoma is a chronic conjunctivitis, and blinding endemic trachoma is a major health problem in developing countries in Asia, Africa, and the Middle East. Both LGV and trachoma infections are considered to be relatively uncommon in the United States, although trachoma is endemic on some U.S. Indian reservations (3).

In recent years, C. trachomatis, serotypes D through K, have received much attention for their role in sexually-transmitted diseases other than LGV. In many industrialized countries, C. trachomatis infections have

TABLE 1. RELATIONSHIP BETWEEN CHLAMYDIAL SEROTYPES AND HUMAN DISEASE

Species	Serotypes	Disease
<u>C. psittaci</u>	Several unidentified serotypes	Psittacosis
<u>C. trachomatis</u>	L-1, L-2, L-3	Lymphogranuloma venereum
<u>C. trachomatis</u>	A, B, Ba, C	Endemic blinding trachoma
<u>C. trachomatis</u>	D, E, F, G, H, I, J, K	Inclusion conjunctivitis, infant pneumonia, nongonococcal urethritis, epididymitis, cervicitis, salpingitis, peritonitis, perihepatitis, endocard- itis, proctitis, acute urethral syndrome in women, pneumonia of immunocompro- mised adults

reached epidemic proportions, and the spectrum of genital infections may closely parallel that of the gonococcus (19,20). C. trachomatis genital-tract infections are observed in both males and females, and the organism in the cervix may be transmitted to the neonate at delivery. Newborns can develop inclusion conjunctivitis and/or a distinctive pneumonia syndrome (21). Prevalence studies indicate that inclusion conjunctivitis is the major eye infection of newborns (19), and that 30% of infants hospitalized with pneumonia are infected with C. trachomatis (22).

C. trachomatis is one of the leading known causes of nongonococcal urethritis (NGU) (23). Chlamydiae have been isolated from 25% to 58% of men with NGU, but only 0% to 7% of men without signs of the disease (24). Epididymitis appears to be the major complication in males infected with non-LGV strains of C. trachomatis (19,25).

Cervicitis is the major genital infection caused by C. trachomatis in females (19). Chlamydial cervical infections are often inapparent, and invasion of the upper genital tract may occur resulting in acute salpingitis (3,26), peritonitis, and perihepatitis (Fitz-Hugh Curtis Syndrome) (24). An acute follicular conjunctivitis may develop in adults if infected genital discharges are spread to the eyes (19).

Individuals with gonorrhea frequently have concomitant chlamydial infections. An average of 20% of men with urethral gonorrhea, and 40-60% of women with cervical gonorrhea have concurrent C. trachomatis infections (27). Penicillin therapy usually is not effective against chlamydiae, and if C. trachomatis is not eradicated, post gonococcal urethritis (PGU) or pelvic inflammatory disease (PID) in females may develop (28). Recommended treatments for C. trachomatis infections would include tetracycline, erythromycin and sulfisoxazole (28).

C. trachomatis may be an opportunistic pathogen of the lower respiratory tract of immunocompromised adults (29,30). A number of other human diseases have been associated with C. trachomatis, including Reiter's syndrome, proctitis, endocarditis, and acute urethral syndrome in women (24,28). An increasing awareness of the prevalence of C. trachomatis, and the continued use of diagnostic testing should establish further etiological roles of chlamydiae in human diseases.

Diagnosis of Chlamydial Infections

The present laboratory methods for diagnosis of chlamydial infections include direct observation of chlamydial inclusions in cytological specimens, testing of patient sera for chlamydial antibodies, and isolation of the organism from clinical specimens (31).

C. trachomatis infections of the conjunctiva, urethra or cervix can be diagnosed by demonstrating typical intracytoplasmic inclusions in cytological specimens stained with Giemsa or immunofluorescent techniques. The immunofluorescent techniques are more sensitive than Giemsa stain for detecting inclusions, but Giemsa-stained specimens can be assessed for inflammation and secondary bacterial infections (3,32,33). Both techniques are effective for diagnosis of trachoma and infant inclusion conjunctivitis, but less than 70% of genital-ocular infections of adults may be detected by direct observation of cytological specimens (3).

Two serological methods available for diagnosis of chlamydial infections are the complement-fixation (CF) test, and the microimmunofluorescence (micro-IF) test (31). The CF test detects antibodies to a group-specific antigen of chlamydiae, and is effective in diagnosis of LGV and psittacosis. The CF test is not sensitive in detecting chlamydial

antibodies in genital-ocular infections where the organisms primarily infect superficial epithelial surfaces (3,33).

The micro-IF test is more sensitive than the CF test in detecting antibodies to C. trachomatis, and this test allows for the determination of the immunoglobulin class of antibody (3,31,34). Serodiagnosis of genital-ocular infections with the micro-IF test is difficult because 23% to 40% of the "normal" adult population have chlamydial antibodies (23,24). Serology can be used to diagnose chlamydial pneumonia of neonates since high levels of IgM antibody are usually found in association with the disease (31).

Direct isolation and identification of chlamydiae from appropriate clinical specimens is the present method of choice for laboratory diagnosis (35). Although all chlamydial strains can be propagated in the yolk sac of developing chick embryos, isolation of the organism in tissue culture cells is a more rapid, sensitive technique (36). The agents of psittacosis and LGV can be grown readily in a variety of cell cultures, but the trachoma-inclusion conjunctivitis (TRIC) strains are more difficult to grow (32). Gordon and co-workers (37) were the first to isolate TRIC strains of C. trachomatis in tissue culture cells. They found that centrifugation of inoculum onto a confluent monolayer of McCoy cells allowed for growth of the organism. The glycogen-containing inclusions of C. trachomatis could be demonstrated by staining the monolayers with iodine after 48 hours of incubation. Gordon and Quan (38) showed that inclusion-formation was enhanced by ultraviolet irradiation of the McCoy cells before the addition of the inoculum.

Various modifications of Gordon's procedure have been developed, but present isolation methods for non-LGV strains of C. trachomatis

share two basic features: the centrifugation of the inoculum to enhance chlamydia-host cell contact; and ultraviolet or chemical treatment of the tissue culture cells to enhance inclusion formation (39).

The centrifugation step in the isolation procedure for C. trachomatis makes it necessary to grow the tissue culture cells on "flying" coverslips in glass vials. The number of inclusions formed in the cell monolayers will increase linearly with the force and duration of centrifugation (39,40,41). Increasing the temperature of centrifugation from 18 to 35°C resulted in a four-fold increase in the number of chlamydial inclusions formed in cell monolayers (41). Optimal isolation rates of C. trachomatis from clinical specimens have been achieved with centrifugation at 2500 to 3000 x g for 1 hour at 33 to 35°C (42).

The effect of irradiation or chemical treatments of tissue culture cells is to produce monolayers of large non-dividing cells that will not overgrow or obscure chlamydial inclusions during a 48 to 72 hour incubation period (39). Iododeoxyuridine (IUdR) (43) cytochalasin B (44), and cycloheximide (45) have been used to treat McCoy cells. Cycloheximide-treated McCoy cells yield significantly more inclusions than those treated with irradiation or other chemical treatments (46,47).

Diethylaminoethyl-dextran (DEAE-dextran) has been used to pretreat tissue culture cells before the addition of the chlamydial inoculum (48,49,50). DEAE-dextran enhances the attachment of non-LGV strains of C. trachomatis, apparently by modifying electrostatic charges between the host cell surface and the chlamydial EB (51,52). DEAE-dextran treatment does not enhance inclusion formation by LGV serotypes of C. trachomatis; this indicates different surface properties among the chlamydial strains (53).

Tissue culture cells other than McCoy's have been used for isolation of C. trachomatis (48,50,54,55). HeLa 229 cells pretreated with DEAE-dextran have been shown to be at least as sensitive to infection as McCoy cells (50). Irradiated and replicating BHK-21 cells have been used successfully for isolation of C. trachomatis, but discrepant results in different laboratories reveal that no one cell line yields the same sensitivity in every laboratory (24,39). Although McCoy cells are the most commonly used, the susceptibility of these cells to infection with C. trachomatis varies with the sub-line of cells utilized (56).

The typical intracytoplasmic inclusions of C. trachomatis are observed by staining the cell monolayers with iodine or Giemsa stains after 48 hours of incubation (31). Chlamydia inclusions can be detected within 24 hours by use of an immunofluorescence stain (57). Recently, monoclonal antibodies have been developed for group and species-specific antigens of chlamydiae, and these antibodies can be labeled with fluorescein or an immunoperoxidase stain (58,59, Immunolok, Inc., Carpinteria, California). Monoclonal immunofluorescent techniques show an increased sensitivity, and an eight-fold and four-fold increase in numbers of inclusions counted when compared with iodine and Giemsa stains, respectively (59,60).

Thus, the sensitivity of the isolation method used can be affected by the staining technique, the cell line and cell treatment utilized, as well as the force, duration and temperature of centrifugation. Other factors that influence the sensitivity of tissue culture isolation of C. trachomatis include the number and condition of the cells explanted, the pH and nutrients contained in the growth medium, and even the serum supplements (39,56,61). Newborn calf serum and certain lots of fetal calf serum have been shown to have deleterious effects on the quantity

and quality of chlamydial inclusions formed in cell monolayers (62,63).

A critical factor in the successful isolation of C. trachomatis from clinical specimens is the proper collection and transport of material to be tested. Cultures of discharges are inadequate; epithelial cells from the infected site must be obtained (3). Swabs or cell scrapings should be extracted into transport medium and kept at 4°C or -70°C because the viability of the organism is lost quickly at room temperature (32). A variety of media have been used for transport of specimens including sucrose phosphate medium (2SP), sucrose-phosphate-glutamate solution (SPG), and tryptose-phosphate broth. The optimal conditions for transport and storage have not been determined (56).

Although the prevalence of C. trachomatis as a sexually-transmitted pathogen is increasing, diagnostic testing is not routinely performed in many clinical laboratories because of the complexity, expense, and problems involved in tissue culture isolation of the organism. A single standardized procedure for diagnostic testing has not been developed since there are a variety of methods used for growth of C. trachomatis in cell cultures, and the literature indicates that no one method has the same sensitivity in every laboratory. The purpose of this study was to determine the most effective and practical method for diagnostic isolation of C. trachomatis in this laboratory. Initially, the possibility of using suspended McCoy cells instead of McCoy cell monolayers for isolation of the organism was examined. Secondly, several types of transport (inoculum-suspending) media were evaluated for optimal transport, storage, and isolation in McCoy cell cultures.

A third aspect of this study was a comparison of staining techniques used for detection of C. trachomatis from clinical samples. Specimens

for this portion of the study were procured from area health facilities, and clinical isolation procedures were carried out to reflect the need for, and the problems encountered in providing this diagnostic service to the community.

MATERIALS AND METHODS

Tissue Cultures

McCoy cells, a heteroploid mouse fibroblast cell line, were the principal cells used for the isolation of C. trachomatis. McCoy cells were purchased from M. A. Bioproducts, Walkersville, Maryland. L (clone 929) cells (American Type Culture Collection, Rockville, Maryland) are a heteroploid mouse fibroblast cell line that was used in an initial study for the isolation of C. trachomatis. Both cell lines were maintained at 36°C in sealed 72 cm² plastic tissue culture flasks (Corning, Corning Glass Works, Corning, New York), and the cells were subcultured at three- to five-day intervals.

For subculture of McCoy cells, the monolayers were washed twice with calcium-magnesium-free phosphate buffered saline (CMF-PBS); then 3 to 5 ml of 0.01% trypsin (Worthington Biochemical Corp., Freehold, New Jersey) in CMF-PBS was added to the flask. The cells were maintained at room temperature for 1 to 2 minutes, the trypsin was decanted, and the cells were incubated at 36°C until they detached from the flask surface. The cells were dispersed by adding 8 to 10 ml of Eagle's minimum essential medium containing Earle's balanced salt solution and L-glutamine (MEME) (M. A. Bioproducts, Walkersville, Maryland) supplemented with 5% fetal bovine serum (FBS) (M. A. Bioproducts, Walkersville, Maryland). The cells were triturated and dispensed to tissue culture flasks containing 20 ml of MEME and 5% FBS. Culture flasks were seeded with approximately $1-2 \times 10^5$ cells per ml to yield full monolayers in 3 to 4 days.

L-929 cells were subcultured by disrupting the monolayer with a

sterile rubber policeman, and triturating with 8 to 10 ml of Medium 199 (M199) containing Hanks' balanced salt solution (Hanks' BSS) (Grand Island Biological Company, Grand Island, New York) supplemented with 10% FBS. Four to five days after subculture, the M199 was replaced with maintenance medium consisting of M199 containing Earle's balanced salt solution (EBSS), supplemented with 5% FBS.

Chlamydia trachomatis Stock Cultures

Chlamydia trachomatis strain UW-36, serotype J, was generously provided by Julius Schachter, Ph.D., George Williams Hooper Foundation, San Francisco, California. The chlamydiae were propagated in McCoy cells and stored at -70°C (Revco Ultra-low Freezer, Revco, Inc., Deerfield, Michigan) at a concentration of 10^4 to 10^6 inclusion-forming units (IFU) per ml in a medium consisting of 50% MEME and 50% 4SP medium containing 3% FBS. 4SP medium consists of 0.4 M sucrose in 0.02 M phosphate buffer, pH 7.2. For experimentation, stock cultures were diluted to yield 50-500 IFU per coverslip culture. Dilutions of chlamydiae were made in 0.2 M sucrose in 0.02 M phosphate buffer (2SP), pH 7.2 unless otherwise stated (64).

Growth Medium

The growth medium used for Chlamydia trachomatis (CGM) consisted of MEME supplemented with 10% FBS, 30 μM glucose, and 20 mM HEPES buffer (M. A. Bioproducts, Walkersville, Maryland). Gentamicin (10 μg per ml) (Grand Island Biological, Grand Island, New York) and amphotericin B (2 μg per ml) (Grand Island Biological, Grand Island, New York) were added to the medium to reduce bacterial contamination of cell cultures (64).

Isolation of *C. trachomatis* in McCoy Cell Monolayer Cultures

Cycloheximide-treated McCoy cell monolayer cultures were used for isolation of *Chlamydia trachomatis* (45,46).

McCoy Cell Monolayer Cultures

McCoy cell monolayer cultures for isolation of *C. trachomatis* were prepared from 3- to 4-day-old cell cultures. The cells were washed with CMF-PBS, and treated with 0.01% trypsin as described previously. The cells were triturated and diluted approximately five-fold in MEME containing 5% FBS. The cells were diluted by adding 1.0 ml of the suspension to 9.0 ml of Isolyse (Coulter Diagnostics, Inc., Hialeah, Florida). Duplicate dilutions were prepared, and the cells were enumerated with a Coulter Hemo-W (Coulter Diagnostics, Inc., Hialeah, Florida). The original cell suspension was adjusted to 5×10^5 cells per ml with MEME and 5% FBS. Monolayers were prepared by adding 1.0 ml aliquots to 1-dram glass shell vials (Kimble, American Scientific Products, McGaw Park, Illinois). The shell vials contained a 12 mm glass coverslip (Bellco Glass, Inc., Vineland, New Jersey). The vials were sealed with silicone stoppers, and incubated at 36°C for use on the following day.

Inoculation and Centrifugation of Cell Cultures

The MEME was removed from the McCoy cell monolayers, and 0.2 ml of *C. trachomatis* stock dilution was added to each vial. The vials were placed in carrier buckets and centrifuged at 500 x g in an International, model HN centrifuge (International Equipment Co., Needham Heights, Massachusetts) for 1 hour at 36°C (Environmental Incubator, Labline, Inc., Chicago, Illinois). The inoculum was removed, and 1.0 ml of CGM containing 0.5 µg of cycloheximide (Sigma Chemical Company, St.

Louis, Missouri) per ml was added. The vials were incubated at 36°C for 48 to 50 hours.

Detection of *C. trachomatis* by Iodine Staining

The CGM was aspirated from the McCoy cell cultures, and 0.5 ml of Jone's iodine (see Appendix B) was added to each vial (56). After standing for 10 minutes at room temperature, the iodine solution was decanted, and the coverslips were removed from the vials with a metal spatula. The coverslips were mounted cell side down in 1 drop of Jone's iodine-glycerin (see Appendix B) on a glass microscope slide. The cell monolayers were examined with a light microscope at 100X magnification. Questionable inclusions were examined at 250-450X magnification for characteristic chlamydial inclusions.

McCoy vs. L-929 Cells for Isolation of *C. trachomatis*

In a preliminary study, L-929 cells were tested as a possible alternative to McCoy cells for isolation of *C. trachomatis*. L-929 monolayer cultures were prepared as described for McCoy cell monolayer cultures, except L-929 cells were triturated and diluted in M199 containing EBSS supplemented with 5% FBS. McCoy and L-929 cell monolayer cultures were prepared on the same day, and after 24 hours of incubation at 36°C, the medium was removed and a 0.2 ml aliquot of *C. trachomatis* stock dilution was added to each culture vial. The cultures were centrifuged, incubated and stained as described for McCoy cell monolayer cultures. The number of inclusions in 20 fields at 400X magnification was determined for ten monolayer cultures for each cell line.

Isolation and Enumeration of *C. trachomatis* in McCoy Cell Suspension Cultures

The effectiveness of using suspended McCoy cells for simplifying the isolation of *C. trachomatis* in tissue culture cells was evaluated. McCoy cell suspensions were prepared by washing and trypsinizing culture-flask cells as described previously. The cells were triturated and diluted in CGM, and counted using a Coulter Hemo-W. Cell counts were adjusted to 2.5×10^5 cells per ml with CGM, and 1.0 ml aliquots were added to 1-dram vials containing a 12 mm coverslip. A 0.2 ml aliquot of *C. trachomatis* stock dilution was added immediately to each vial. The suspensions were mixed gently and centrifuged at 500 x g for 1 hour at 36°C. The cells were incubated for 24 hours at 36°C; the CGM was removed, and 1.0 ml of CGM containing 0.5 µg/ml cycloheximide was added to each vial. The cultures were incubated for an additional 24 hours at 36°C.

The suspension cultures were stained with Jone's iodine, and the coverslips mounted in Jone's iodine-glycerin on glass microscope slides. The number of inclusions in the suspension cultures was compared to the numbers found in McCoy cell monolayer cultures inoculated with an equivalent sample of the chlamydial dilution. Inclusions per coverslip were determined using 100X magnification.

Evaluation of Temperature and Centrifugal Force Used for *C. trachomatis* Isolation

The maximum centrifugal force attainable with the International, model HN centrifuge (International Equipment Co., Needham Heights, Massachusetts) and the Damon IEC, model HNS II, centrifuge (International

Equipment Co., Needham Heights, Massachusetts) used in this laboratory was 900 x g. When centrifugation was performed at 36°C, increases in force (> 500 x g) resulted in increased temperatures within the vials (> 40°C).

A comparison of the recovery of chlamydial inclusions with centrifugation at 500 x g for 1 hour at 36°C, and centrifugation at 900 x g for 1 hour at room temperature was performed. McCoy cell monolayer cultures were inoculated with 0.2 ml of C. trachomatis stock dilution, and sets of vials were centrifuged under both conditions. The inoculum was removed from the monolayers and CGM with 0.5 µg/ml cycloheximide was added to each vial. The culture vials were incubated for 48 hours at 36°C; the monolayers were stained with iodine, and the number of chlamydial inclusions per coverslip was determined.

Effect of Inoculum-Suspending Medium on the Recovery of C. trachomatis

Four inoculum-suspending (transport) media were evaluated for their effects on the recovery of C. trachomatis in McCoy cell monolayers. The media tested were 0.2 M sucrose-phosphate medium (2SP), sucrose-phosphate glutamate solution (SPG) (see Appendix B), Hanks' balanced salt solution (Hanks' BSS) (Flow Laboratories, Rockville, Maryland), and tryptic soy broth (T-soy broth) (Difco Laboratories, Detroit, Michigan). The 2SP, SPG, and Hanks' BSS were supplemented with 3% FBS. Gentamicin and amphotericin B were added to each medium to give a final concentration of 10 µg/ml and 4 µg/ml, respectively.

Equivalent dilutions of C. trachomatis stock organism were prepared in each medium, and 0.2 ml of each were inoculated onto McCoy cell monolayer cultures. The culture vials were centrifuged at 900 x g for 1

hour at room temperature. The inoculum was aspirated from the monolayers, and CGM with cycloheximide was added to each vial. After 48 hours' incubation at 36°C, the monolayers were stained with iodine, and the number of inclusions per coverslip was determined.

Storage at 4°C

The four inoculum-suspending media were evaluated for their ability to maintain the viability of C. trachomatis at 4°C. Equivalent dilutions of the C. trachomatis stock organism were prepared in each medium and stored at 4°C. Each medium was tested daily in McCoy cell monolayer cultures for the number of IFU remaining per sample over a three-day period.

Storage at -70°C

The four media and 2SP supplemented with 10% dimethyl sulfoxide (DMSO) (Sigma Chemical Co., St. Louis, Missouri), and 2SP supplemented with 10% glycerol (Mallinckrodt Chemical Works, St. Louis, Missouri), were tested for their effects on the viability of C. trachomatis after storage at -70°C. Equivalent dilutions of the C. trachomatis stock organism were prepared in each medium, and the number of inclusions formed in McCoy cell monolayer cultures was determined at zero time and after one freeze-thaw cycle at -70°C.

To determine the effect of long-term storage at -70°C on the viability of C. trachomatis, several 1.0 ml aliquots of 2SP containing the C. trachomatis stock organism were stored at -70°C for a six-week period. The 2SP samples were tested at 48 hours, 6 days, and 6 week intervals for the number of viable organisms remaining per sample.

Comparison of Techniques for the Detection of C. trachomatis from Clinical Specimens

Four area health facilities provided the clinical specimens used to compare the effectiveness of iodine, immunoperoxidase, and immunofluorescence staining procedures for the detection of chlamydial inclusions in McCoy cell monolayer cultures. The format used for this study with human subjects was approved by the Institutional Review Board, Office of Research and Program Development, University of North Dakota.

Collection and Transport of Specimens

The specimens were collected by the physicians or nursing staff at each health facility. Cells from the endocervical or urethral canal were removed with a swab (Culturette, Marion Scientific, American Scientific Products, McGaw Park, Illinois), or a curette (Cyto-Spatula, Lab-Tek, Curtin Matheson Scientific, Inc., Houston, Texas) and extracted into 2SP medium containing 3% FBS, gentamicin, and amphotericin B. The samples were kept at 4°C; or if the transport time exceeded 24 hours, the specimens were to be frozen at -20°C for storage and transport. The specimens received in this laboratory were frozen at -70°C until tested.

Isolation Procedure

The frozen samples were thawed quickly by placing the transport tubes at 35°C for 2 to 3 minutes. Sterile glass beads were added to each transport tube and the tubes were vortexed for 10 to 15 seconds. Any extensively turbid sample was centrifuged for 10 minutes at 300 x g. Four McCoy cell monolayer cultures were used per specimen, and 0.2 ml of sample-supernatant was inoculated into each vial. The remaining portion of the clinical specimen was stored at -70°C. The culture vials

were centrifuged at 900 x g for 1 hour at room temperature. The inoculum was removed, and 1.0 ml of CGM containing 0.5 µg/ml of cycloheximide was added. The cell monolayers were stained after 48 hours of incubation at 36°C. Two monolayer cultures per sample were stained with Jone's iodine, and two were tested for C. trachomatis by the immunoperoxidase assay.

Immunoperoxidase Assay

The reagents for the immunoperoxidase assay were purchased in a kit (Chlamydia Identification, PAP) from Immulok, Inc., Carpinteria, California.

The McCoy cell monolayers were washed with PBS, then fixed with cold absolute methanol for ten minutes. The methanol was removed, and the remaining reagents were added either directly to the culture vials, or the coverslips were removed and placed cell side up on a 12-well serological glass slide. The latter method was used to conserve on the amount of reagents used for this assay. For staining the coverslips in the vials, 5 drops of each reagent was added per vial. Only 2 to 3 drops of reagent were used per coverslip placed on the serological slide. To prevent drying of the coverslips during application of the antibody reagents, the serological slide was placed in a humidity chamber consisting of a 150 x 15 mm plastic Petri plate (Falcon Labware Division, Becton, Dickinson and Co., Oxnard, California) containing damp paper towels.

Three antibody reagents were employed in this assay. Each reagent was added separately for incubation at room temperature for 20 to 30 minutes. All coverslips were washed with PBS between the application of the reagents. The primary antibody (mouse monoclonal antibody to

chlamydiae) was added first. The linking reagent (goat anti-mouse IgG) was the second antibody applied, followed by the labeling reagent (the peroxidase mouse-antiperoxidase antibody or PAP).

If chlamydial inclusions were present in the cell monolayers, the binding of the three antibodies was demonstrated by the addition of a chromagen substrate. The substrate reagent contains 1 drop of 3-amino-9-ethylcarbazole (AEC) in N-N-dimethyl formamide, and 1 drop of 1% hydrogen peroxide in 2.0 ml acetate buffer. The substrate chromagen reagent was allowed to react with the bound enzyme for 40 minutes at room temperature. The cell monolayers were counterstained for 3 minutes with Meyer's hematoxylin. The coverslips were placed cell side down in 1 drop of aqueous mounting medium on a glass microscope slide.

The coverslips were examined with a light microscope at 100X magnification. Chlamydiae were visible as reddish-brown intracytoplasmic inclusions against a blue background of cells. Questionable inclusions were examined at 450X magnification for typical morphology.

Immunofluorescence Assay

The clinical specimens that were found to be positive for C. trachomatis were tested a second time to compare the effectiveness of the immunofluorescence assay to iodine staining. Four McCoy cell monolayer cultures were infected per sample as previously described. Two monolayer cultures were stained with Jone's iodine, and two were stained with an indirect fluorescent antibody technique.

The reagents for the immunofluorescence assay were purchased from Immulok, Inc., Carpinteria, California (Chlamydia Identification, IFA). The cell monolayers were washed with PBS, and fixed with absolute methanol for ten minutes at room temperature. Five drops of mouse monoclonal

antibody to Chlamydia were added to each vial, and the cultures were incubated at room temperature for 30 minutes. The antibody reagent was removed and the monolayers were washed with PBS. Five drops of FITC-conjugated goat anti-mouse IgG reagent were added to each vial and the cultures were incubated for 30 minutes at room temperature. The monolayers were washed with PBS, and the coverslips were removed from the vials and mounted in aqueous mounting medium on glass microscope slides.

The coverslips were examined with a Leitz Ortholux microscope fitted with a high-power darkfield condenser (Ernst Leitz, Wetzlar, Germany). For transmitted light fluorescence, a high-pressure mercury vapor lamp was used with a BG12 excitation filter, a BG38 red suppression filter, and K510-K530 barrier filters. The coverslips were scanned at 250X magnification, and chlamydiae were observed as large apple-green fluorescent inclusions.

Quantitation of Results

For all staining techniques the number of chlamydial inclusions per coverslip was determined. One typical intracytoplasmic inclusion per clinical specimen was considered positive for Chlamydia.

Quality of Staining Techniques

Photomicrographs were taken of infected monolayer cultures stained with iodine, peroxidase-anti-peroxidase (PAP) and immunofluorescent antibody (IFA) stains. A Leitz Ortholux microscope with an Orthomat microscope camera (Ernst Leitz, Wetzlar, Germany) was used to take photomicrographs at 100X to 400X magnification. The film used for lightfield microscopy was Kodak Ektachrome 64 (daylight) film (Eastman Kodak Co.,

Rochester, New York). For darkfield photomicrographs, Kodak Ektachrome 400 (daylight) film was used.

RESULTS

Comparison of McCoy and L-929 Cells for Isolation of C. trachomatis

Schachter and Dawson (35) report that L-929 mouse fibroblasts provide essentially the same results as McCoy cells for the isolation of C. trachomatis, but studies from other laboratories are not consistent with their findings (40,50). The numbers of chlamydial inclusions formed in McCoy and L-929 cell monolayer cultures were compared to determine if L-929 cells were as effective as McCoy cells for the isolation of C. trachomatis. The mean number of inclusions counted in 20 fields from ten separate cell monolayer cultures was 86 ± 12 for McCoy cells, and only 15 ± 5 for L-929 cells. Less than 20% of the inclusions enumerated in McCoy cells were recovered in similarly treated L-929 cells. This preliminary data indicated that L-929 cells would not yield the same results as McCoy cells for isolation of C. trachomatis in this laboratory. No further testing was performed with L-929 cells.

Recovery of C. trachomatis from McCoy Cell Monolayer and Suspension Cultures

The isolation of C. trachomatis in McCoy cells would be simplified by inoculation of suspension cultures, thus eliminating the need for preformed monolayers. Studies were initiated to determine the feasibility of using McCoy cell suspension cultures as an alternative to monolayer cultures. The recovery of C. trachomatis from McCoy cell monolayer and suspension cultures was evaluated in five separate trials (Table 2). The percent recovery in the suspension cultures as compared to the monolayers varied from 13 to 86%, but the recovery rates were very high (75

TABLE 2. RECOVERY OF CHLAMYDIA TRACHOMATIS FROM MCCOY CELL MONOLAYER AND SUSPENSION CULTURES

Experiment	McCoy Cell Monolayer Cultures	McCoy Cell Suspension Cultures*
1	97±6**	73±28(75)***
2	286±46	116±42(41)
3	136±7	115±10(85)
4	147±23	127±36(86)
5	401±52	52±24(13)

* 2.5×10^5 cells per ml CGM

** Mean number of inclusions from 3 coverslips

*** Percent recovery as compared to McCoy cell monolayer cultures

to 86%) in experiments, #1, #3, and #4. Additional studies for improving the recovery rates of C. trachomatis in McCoy cell suspension cultures were performed. However, the suspended cells were found to be more susceptible to adverse effects from bacterial contamination, toxic products, and temperature variations than the cell monolayers. The differences between the two methods indicated that cell monolayers were more effective than cell suspension cultures for the isolation of C. trachomatis, and monolayer cultures were used for the duration of this study.

Effect of Centrifugal Force and Temperature on the Recovery of C. trachomatis

Increasing the temperature and force of centrifugation can increase the number of chlamydial inclusions recovered in cell monolayers (40, 41,42). Experiments initially were performed at 500 x g for 1 hour at 36°C. Increasing the centrifugal force (> 500 x g) resulted in increased temperatures within the vials (> 40°C). A comparison of the effect of centrifugal force and temperature on the recovery of C. trachomatis from McCoy cell monolayers was performed (Table 3). The number of inclusions formed at 500 x g at 36°C was nearly double the number of inclusions present in monolayers centrifuged at 900 x g at room temperature. Although more chlamydial inclusions were recovered at a higher temperature with a lower speed, the increases in temperature within the centrifuge could not be controlled at 36°C, so centrifugation for the duration of the study was performed at room temperature at 900 x g.

TABLE 3. EFFECT OF CENTRIFUGAL FORCE AND TEMPERATURE ON THE RECOVERY OF C. TRACHOMATIS FROM MCCOY CELL CULTURES

Experiment	500 x g 36°C	900 x g 24°C
1	169±25*	108±6
2	84±24	43±1
3	535±115	280±45

*Mean number of inclusions from 3 coverslips

Evaluation of Inoculum-Suspending (Transport) Medium Used for *C. trachomatis*

Several types of inoculum-suspending (transport) media were evaluated to determine the optimal conditions for transport, storage and inoculation of McCoy cells for isolation of *C. trachomatis*

Effect of Inoculum-Suspending Medium on the Survival of *C. trachomatis* Stored at 4°C

C. trachomatis stock dilutions were stored in 2SP, SPG, Hanks' BSS, and T-soy broth for three days at 4°C. The number of inclusion-forming units present in each medium was determined in McCoy cell monolayer cultures at daily intervals (Figure 1). The recovery of viable organisms decreased by approximately 20% each day when stored in 2SP medium. The loss of viability of *C. trachomatis* was greater in the other three media (24-30% daily), and after 72 hours, the percent recovery of IFU from 2SP was nearly double that of Hanks' BSS, T-soy broth and SPG.

Effect of Inoculum-Suspending Medium on the Recovery of *C. trachomatis*

In the previous experiment, the recovery of chlamydial inclusions at the 0 hour was not equivalent in the four media tested. The effects of suspending *C. trachomatis* in 2SP, SPG, T-soy broth, and Hanks' BSS on the recovery of the organism in McCoy cell monolayer cultures were determined in three separate trials (Table 4). The highest number of IFU consistently were seen when 2SP was used as the inoculum-suspending medium. The recovery rates were reduced by approximately 25% by suspending the organisms in SPG. The use of Hanks' BSS and T-soy broth as inoculum-suspending media decreased the efficiency of inclusion formation

Figure 1. Effect of inoculum-suspending medium on the survival of C. trachomatis stored at 4°C. Values represent the percent recovery from the 0 hour using the mean number of inclusions from three coverslips. Symbols: Inoculum-suspending medium - 2SP (▲—▲); SPG (●—●); T-soy broth (○---○); Hanks' BSS (□---□).

PERCENT RECOVERY OF C. trachomatis

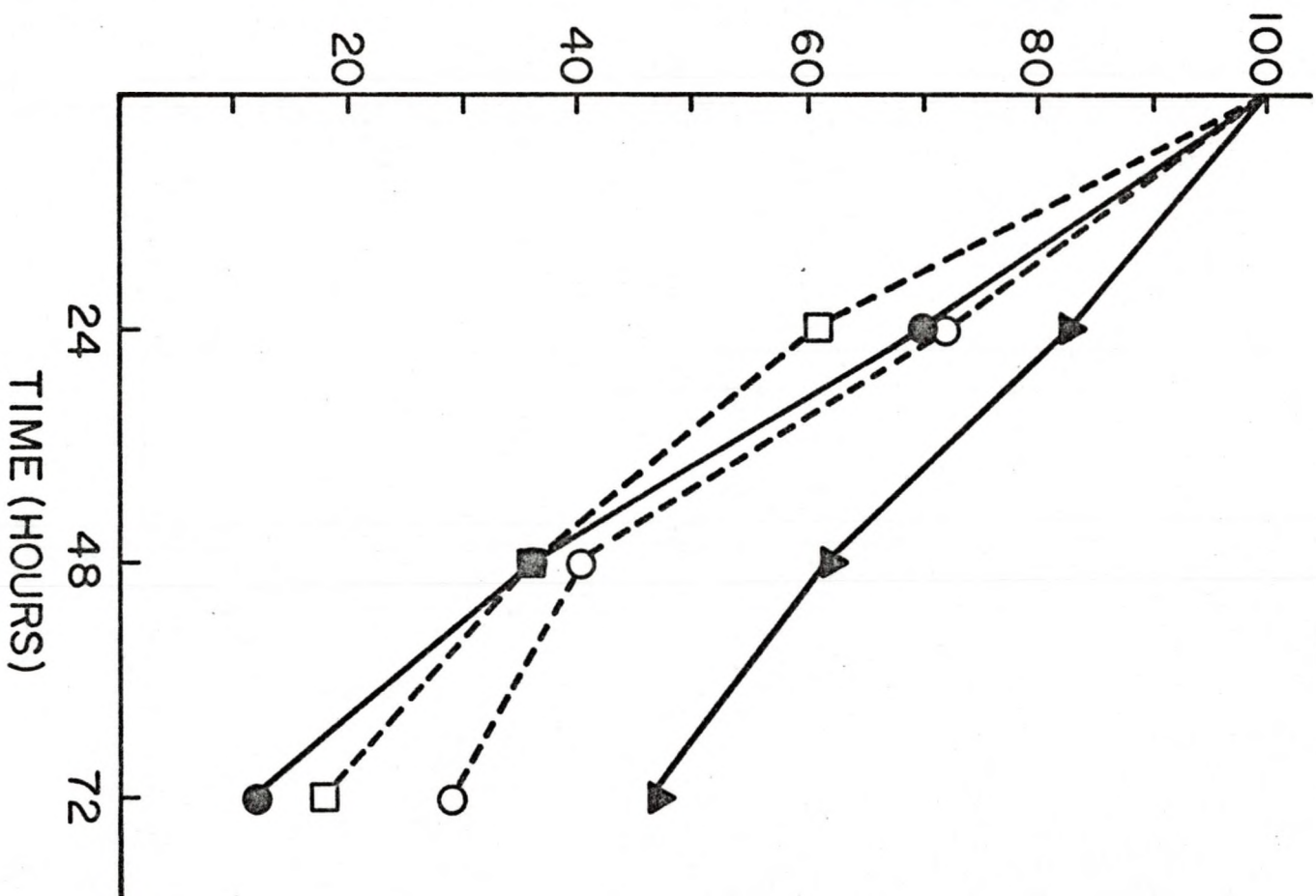


TABLE 4. EFFECT OF INOCULUM-SUSPENDING MEDIUM ON THE RECOVERY OF C. TRACHOMATIS
FROM MCCOY CELL MONOLAYER CULTURES

Experiment	Inoculum-Suspending Medium			
	2SP	SPG	T-soy Broth	Hanks' BSS
1	269±40 [*]	208±9 ^{**}	147±12	150±34
2	284±32 ^{***}	186±42	145±40	Not Done
3	611±7 ^{**}	530±51	475±75	324±21

^{*}2SP controls tested each day, mean of 9 coverslips

^{**}Mean of inclusion counts from 3 coverslips

^{***}Mean of inclusion counts from 4 coverslips

by nearly one-half the number of inclusions recovered from 2SP medium. The results show that the inoculum-suspending medium can affect the number of chlamydial inclusions formed in McCoy cell monolayer cultures.

Effect of Inoculum-Suspending Medium on the Recovery of *C. trachomatis* Stored at -70°C

The loss of viability of *C. trachomatis* after freezing and thawing was determined for six different inoculum-suspending media. 2SP, SPG, Hanks' BSS, T-soy broth, 2SP with 10% DMSO, and 2SP with 10% glycerol were compared for the number of inclusions formed in McCoy monolayer cultures before freezing, and after storage at -70°C for at least 48 hours (Table 5). When compared to the recovery of *C. trachomatis* in 2SP prior to freezing, the recovery rates were decreased by more than 75% in all media tested after one freeze-thaw cycle. The most marked decline in viability was observed with the T-soy broth and Hanks' BSS.

The numbers of inclusions obtained from cultures inoculated with 2SP with DMSO and 2SP with glycerol were essentially the same before and after freezing. Although the addition of DMSO and glycerol to 2SP may aid in maintaining the viability of *C. trachomatis* at freezer temperatures, these supplements appear to interfere with either the uptake or inclusion formation of *C. trachomatis*.

The effects of prolonged storage on the recovery of *C. trachomatis* in 2SP at -70°C were assessed in three separate trials (Table 6). The number of chlamydial inclusions formed in McCoy cell monolayers was determined prior to freezing, and after 48 hours, 6 days, and 6 weeks of storage at -70°C . Although the recovery rates varied from 46 to 85%, the results indicate that no significant loss of organisms occurred other than during the initial freeze-thaw cycle.

TABLE 5. EFFECT OF INOCULUM-SUSPENDING MEDIUM ON THE RECOVERY OF C. TRACHOMATIS AFTER ONE FREEZE-THAW CYCLE AT -70°C

Inoculum-Suspending Medium	0 Hour	-70°C
2SP	$611 \pm 7^*$	$139 \pm 22 (23)^{**}$
SPG	530 ± 51	$145 \pm 23 (24)$
T-soy broth	475 ± 75	$13 \pm 4 (2)$
Hanks' BSS	324 ± 21	$10 \pm 1 (2)$
2SP with DMSO ^{***}	41 ± 10	$46 \pm 4 (16)$
2SP with glycerol ^{***}	44 ± 20	$30 \pm 7 (10)$

* Mean number of inclusions from 3 coverslips

** Percent recovery as compared to 2SP control at 0 hour

*** 2SP control, mean number of inclusions from 3 coverslips was 296 ± 74

TABLE 6. EFFECT OF STORAGE AT -70°C ON THE RECOVERY OF C. TRACHOMATIS IN 2SP MEDIUM

Experiment	Period of Storage at -70°C in 2SP			
	0 Hour	48 Hours	6 Days	6 Weeks
1	296±13*	164±54(55)**	141±16(47)	157±12(53)
2	296±74	183±32(62)	252±49(85)	137±14(46)
3	183±10	119±10(65)	105±10(57)	Not Done

*Mean number of inclusions from 3 coverslips

**Percent recovery as compared to 2SP control at 0 hour

Comparison of Staining Techniques for the Detection of *C. trachomatis*

Clinical specimens were used to compare the effectiveness of the immunoperoxidase (PAP) assay and iodine staining for the detection of *C. trachomatis* in McCoy cell monolayer cultures (Table 7). Of 139 specimens tested, 12 (9%) were positive by iodine staining, with 7 (5%) positive by the PAP assay. The sensitivity of the PAP assay was lowest in specimens containing fewer than 20 IFU per coverslip by iodine staining. In these latter specimens, only 1 of the 6 specimens positive by iodine staining was also positive by the PAP assay. In specimens containing more than 20 IFU per coverslip, the number of positive specimens was the same for both stains.

Ten positive samples were stored at -70°C , and tested a second time with the immunofluorescence assay (IFA) and iodine stain. Only eight samples were positive with iodine staining, and seven were positive by IFA. Each of the two specimens that were no longer positive for Chlamydia had only one IFU per coverslip on the first staining. The one sample positive by iodine staining but not IFA had only one IFU on one coverslip.

Iodine-stained chlamydial inclusions are visible at 100X magnification as large brown intracytoplasmic inclusions against a yellow background of cells (Figure 2A). Epithelial cells are stained with iodine, but these can be readily distinguished from chlamydial inclusions at 100X to 450X magnification (Figure 2B).

Chlamydial inclusions stained by the PAP assay are dark red intracytoplasmic inclusions against a blue background of cells (Figure 3A). Inclusions can be detected at 40X magnification, but because of non-specific staining of monolayers, coverslips were scanned at 100X

TABLE 7. COMPARISON OF CHLAMYDIA-POSITIVE SPECIMENS DETECTED BY IODINE STAINING AND IMMUNOPEROXIDASE (PAP) ASSAY

Stain	Number of Positive Specimens (n=139)**	Comparison of Positive Specimens By Numbers of IFU Per Monolayer*		
		< 20	20-100	> 100
Iodine	12	6	2	4
PAP	7	1	2	4

* Mean number of inclusion-forming units from two coverslips

** Number of specimens tested

magnification. Nonspecific and background staining (precipitates) occurred frequently with the PAP assay (Figure 3B). Epithelial cells would stain red, and when numerous epithelial cells were present, the entire monolayer would be red colored.

In the IFA assay, chlamydial inclusions were large with dense fluorescence that usually was bound by an inclusion membrane (Figure 4A). Nonspecific staining was seen in cell monolayers, especially if epithelial cells or bacteria were present (Figure 4B).

Figure 2A. Iodine-staining of chlamydial inclusions in McCoy cell monolayer cultures. 100X magnification.

2B. Iodine-staining of chlamydial inclusions in McCoy cell monolayer cultures. 400X magnification.

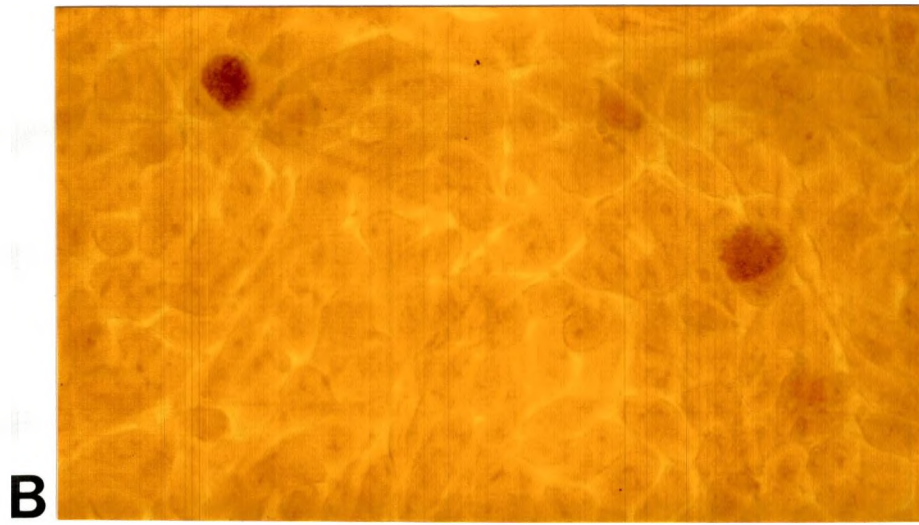
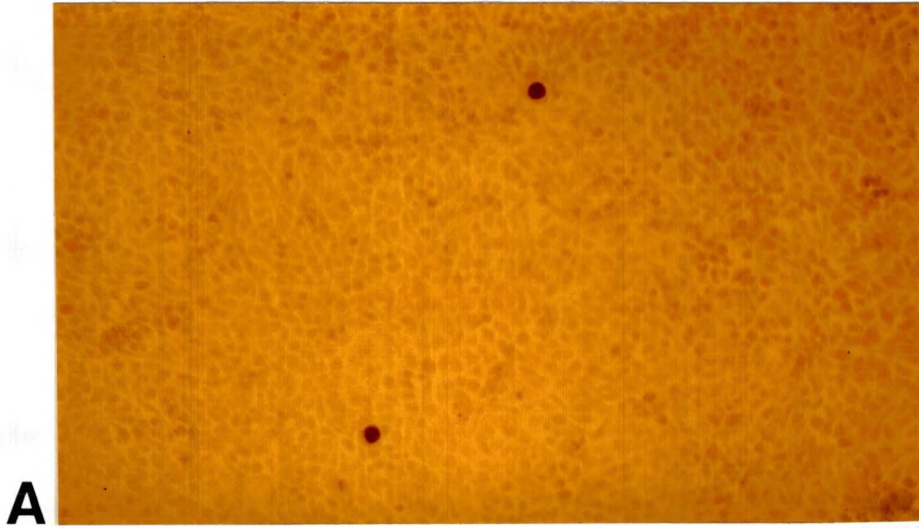
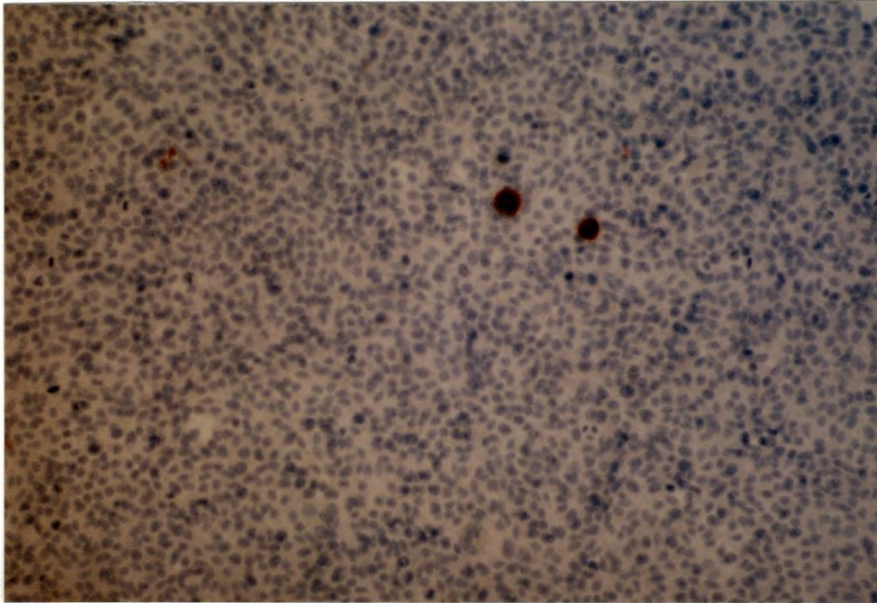
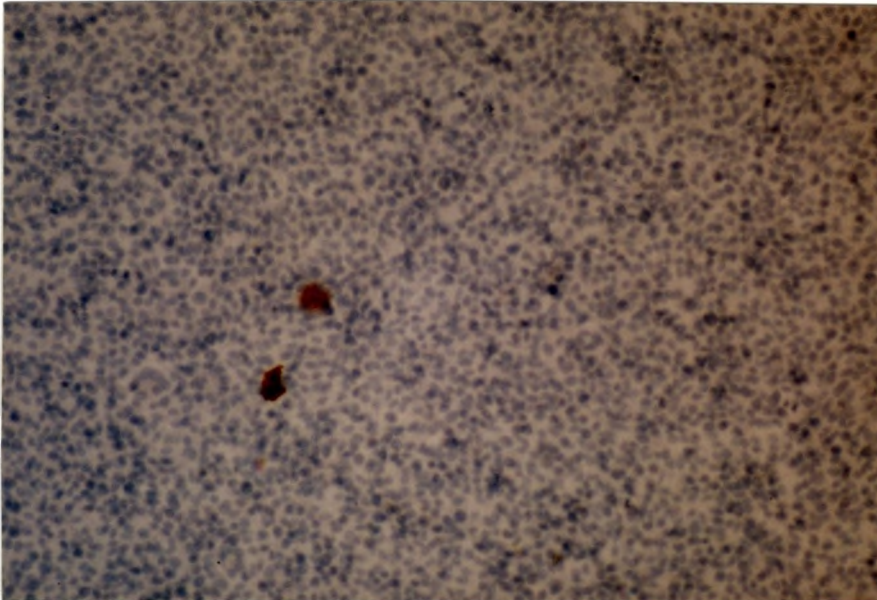


Figure 3A. Immunoperoxidase (PAP) staining of chlamydial inclusions in McCoy cell monolayer cultures. 100X magnification.

3B. Nonspecific staining of McCoy cell monolayers with the PAP assay. 100X magnification.



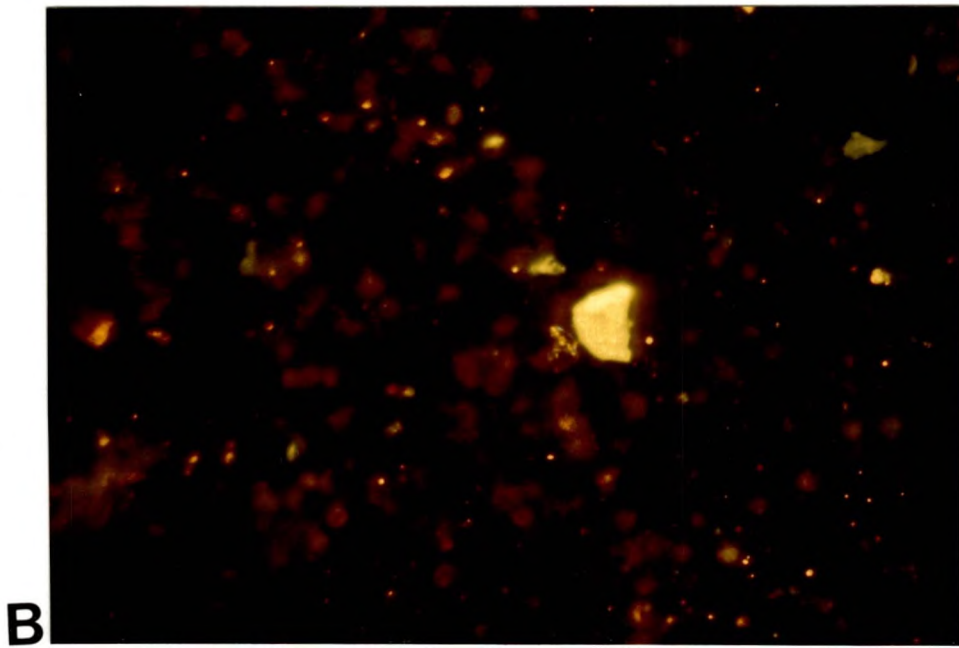
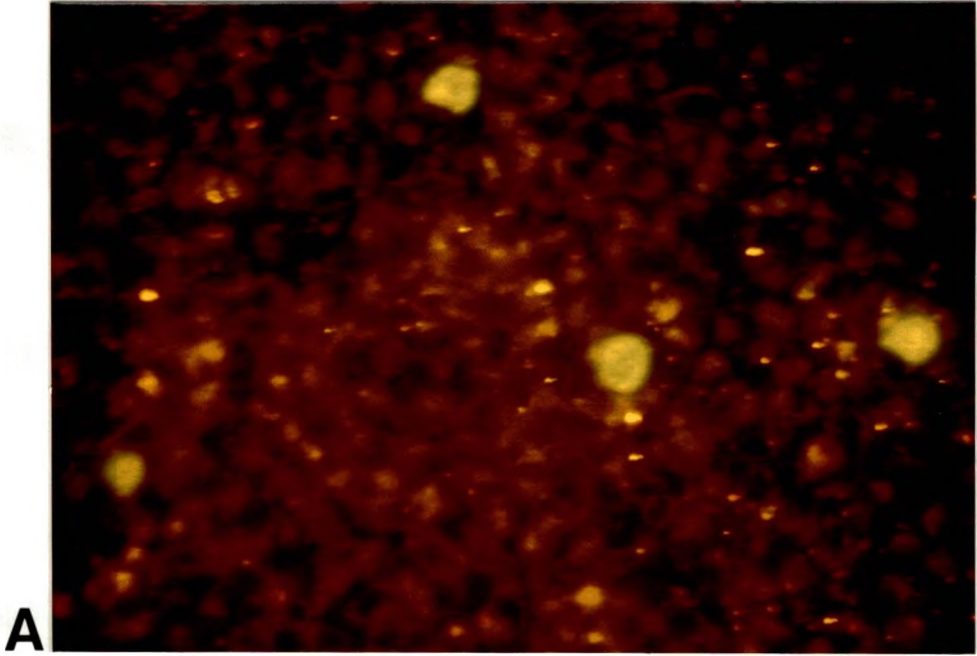
A



B

Figure 4A. Immunofluorescent (IFA) staining of chlamydial inclusions in McCoy cell monolayer cultures. 250X magnification.

4B. Nonspecific staining of McCoy cell monolayers with the IFA assay. 250X magnification



DISCUSSION

In recent years, Chlamydia trachomatis has been shown to be a significant pathogen of sexually-active adults, and of newborns. The growing prevalence of infections caused by C. trachomatis indicates the need for a rapid, sensitive method for diagnosis. Although tissue culture isolation of the organism is presently the method of choice, this procedure is not routinely available in most clinical laboratories because of the complexity, expense, and difficulties involved. Various parameters of tissue culture isolation of C. trachomatis were investigated in this study to determine the feasibility of providing this diagnostic service to area health facilities.

Before implementation of a laboratory diagnostic test, consideration must be given to the effectiveness, the costs, the problems that may be encountered, and the necessity of performing this procedure. In terms of effectiveness, the overall sensitivity of cultural methods for isolation of C. trachomatis is unknown, although estimates of 70-80% have been given for the detection of chlamydial urethritis and cervicitis (28). Conflicting reports in the literature indicate that the sensitivity of tissue culture isolation will vary among laboratories, and this sensitivity can be affected by a number of different factors. Major determinants of the effectiveness of cultural methods for C. trachomatis include the line or subline of cells utilized, the use of chemical treatments, the conditions of centrifugation, and the staining technique used to detect chlamydial inclusions. Also, no clinical isolation method can be effective without the proper

collection and transport of specimens to be tested.

The first consideration was given to the choice of cell line. McCoy cells are the most widely employed cell line for isolation of C. trachomatis. However, the origin and characterization of McCoy cells is questionable, and these cells have become increasingly heterogeneous because of a varying passage history in a number of different laboratories (65). Efforts have been made to encourage research on C. trachomatis with a more defined cell line such as L (clone 929) cells. L-929 cells are a heteroploid mouse fibroblast cell line also, and have been reported to be as sensitive as McCoy's for isolation of C. trachomatis (35). Preliminary studies in this laboratory did not reflect this sensitivity, and only 20% of the inclusions recovered in McCoy cell monolayer cultures were recovered in similarly treated L-929 cells.

The use of any rapidly growing cell such as McCoy's, requires the use of antireplicative agents to prevent overgrowth of chlamydial inclusions. Treatment with irradiation or IUdR must be done prior to the formation of the monolayer, which adds an additional step to the procedure. Cycloheximide-treatment was chosen because of its reported superiority, and because it can be added directly to the chlamydia growth medium (46). The optimal concentration of cycloheximide initially was reported to be 2 μg per ml (45). However, we found this level to be toxic to McCoy cells. The use of cycloheximide at 0.5 μg per ml concentration maintained the confluency of the monolayer with no visible toxic effects to the cells. Similar findings have been reported in other laboratories (31,56).

The optimal concentration of cells needed to produce confluent monolayers of McCoy cells after one day of incubation was 5×10^5 cells

per ml. Some investigators report that cell concentrations at one-half (2.5×10^5 cells/ml), or one-quarter (1.25×10^5 cells/ml) this number yield confluent monolayers in two and three days, respectively (56,66). Also, confluent monolayer cultures have been held at 4°C or room temperature for several days to maintain the cells until the time of use (3, 56). This latter procedure eliminates the need to prepare cultures several times during a week. However, these techniques did not prove successful in this laboratory, and the McCoy cells detached from the coverslip usually after two to three days.

McCoy cell suspension cultures were proposed as a means of simplifying tissue culture isolation of C. trachomatis. The possible advantages of using suspension cultures were threefold: 1) the number of cells seeded could be adjusted to eliminate the need for cycloheximide; 2) the cultures could be prepared on the day of use, eliminating the time needed for preparation of monolayers; and 3) suspending the cells could free receptors on the cell surface, thus increasing the attachment of chlamydial EB to the host cell.

Seeding McCoy cells at one-half (2.5×10^5 cells/ml) the number needed to yield a full monolayer in 24 hours, resulted in slight overgrowth of the cells at 48 hours. Chlamydial inclusions were easier to detect when the growth medium was changed after 24 hours of incubation to growth medium (CGM) containing cycloheximide. The use of suspended cells at this concentration did not eliminate the need for antireplicative agents; but changing the medium at 24 hours did not add an additional step to the procedure since this must be done with the monolayer cultures after centrifugation anyway.

The efficiency of chlamydial infection of McCoy cells has been

shown to be greater with suspended cells than with monolayer cultures in the absence of centrifugation (67). Only small numbers of inclusions were recovered without centrifugation, so we compared the efficiency of chlamydial infection of suspension cultures vs. monolayer cultures aided by centrifugation. When centrifugation was not performed, no inclusions were recovered in either monolayers or suspensions with the infective dose between 50-500 IFU per coverslip.

In the five trials shown (Table 2) more inclusions were detected in the monolayer cultures, however the recovery rates of chlamydiae in suspension cultures were usually high (75-86% in three trials), and showed potential for further investigation.

Inclusion formation has been shown to be enhanced by pretreatment of cell monolayers with DEAE-dextran (49,51,52,53). Pre-treatment of McCoy cells with DEAE-dextran also increased the efficiency of C. trachomatis infection in suspended cells in the absence of centrifugation (67). In an effort to improve the efficiency of C. trachomatis infection in suspended McCoy cells aided by centrifugation, DEAE-dextran was added to 2SP medium to a final concentration of 30 µg per ml. The chlamydial inoculum containing the DEAE-dextran was added directly to the suspended cells until the media change at 24 hours. Initially, the DEAE-dextran appeared to improve the recovery rates of C. trachomatis, but the polycation produced considerable toxic effects to the cells. DEAE-dextran was toxic to suspended cells when concentrations of 10, 20, and 40 µg per ml were tested. No effects from the 2SP containing DEAE-dextran were observed in McCoy cell monolayer cultures, probably because the inoculum was removed immediately after centrifugation.

The same was true of the presence of bacteria in the

inoculum-suspending medium. Small amounts of bacterial contamination could nearly destroy the suspended cells, yet leave monolayers unaffected. Although clinical samples were never tested in suspended McCoy cells, the evidence indicates that bacteria, mucins, and other toxic products found in patient samples would have the same detrimental effects. These products could not be removed from the suspension cultures, as in the monolayers, because the cells were not completely attached to the coverslips immediately after centrifugation. Also, the increased susceptibility of suspended cells to adverse conditions was shown when centrifugal forces were increased in order to improve the recovery of C. trachomatis after centrifugation at 36°C. The increased temperatures within the centrifuge destroyed the suspended cells, but only the outer edges of the monolayer cultures were affected.

Some of the difficulties encountered with suspension cultures may have been overcome by trying various alternatives such as treating clinical samples with high concentrations of antibiotics to reduce problems with bacterial contamination. Also, changing the medium on the suspension cultures earlier than 24 hours may have helped to eliminate problems with toxic products. These tests were not performed because overall the McCoy cell monolayer cultures were more stable and more effective than McCoy cell suspension cultures. So McCoy cell monolayer cultures were used for the duration of this study.

The optimal conditions for centrifugation of clinical specimens for isolation of C. trachomatis in McCoy cell monolayers have been shown to be 2500 to 3000 x g for 1 hour at 35°C (42). Problems relating to obtaining these optimal conditions are not unique to this laboratory (41,56,68). Centrifugal forces from 2500 to 3000 x g are difficult to

achieve with bench top centrifuges, and some laboratories may have to compromise sensitivity for centrifugal forces at 900 to 1000 x g (56, 68). Equally difficult for many clinical laboratories would be having the equipment and facilities for centrifugation at 33 to 37°C. This is reflected in studies where centrifugation was performed at 2500 to 3000 x g at room temperatures rather than at 33 to 37°C (47,50,57). However, the preliminary results of this study indicate that at lower speeds (< 1000 x g) the temperature of centrifugation may be more critical for optimal recovery of chlamydiae than the force applied. Further tests at higher speeds were not performed because of the limitations of the centrifuges used. Although floor model centrifuges could have been employed to overcome the problems of heating, the conditions of centrifugation used in this study (900 x g at room temperature) were the most practical, and probably quite similar to conditions obtainable in many clinical laboratories. In isolating C. trachomatis from clinical specimens, the effects of temperature and force used for centrifugation would be most significant with samples containing low numbers of inclusion-forming units. Since the clinical specimens used in this study were not tested in parallel by a reference laboratory, there is no definitive way to determine the numbers of Chlamydia-positive specimens missed by using less than optimal conditions for centrifugation. However, prevalence studies done in the United States show isolation rates of C. trachomatis from persons visiting venereal disease clinics to be approximately 12 to 15%, and from the "normal" population, isolation rates of C. trachomatis are about 2 to 3% (personal communication, Dr. Bryon Keihl, Immulok, Inc., Carpinteria, CA). From 139 specimens tested, 74 were from patients with possible genital

tract infections; 10 (14%) of these samples were positive for C. trachomatis. The remaining 72 samples were from patients presenting without signs of disease; 2 (3%) samples from this group were positive for C. trachomatis. Although large numbers were not tested, these values give some assurance that the isolation rates of C. trachomatis in this study were similar to results obtained in other laboratories.

Since this laboratory provides diagnostic services to a number of health facilities in a large area, the problems of transport and storage of clinical specimens tested for C. trachomatis are important concerns. The optimal conditions have not been elucidated, but the rapid loss of viability of the organism at room temperature indicates that immediate inoculation of cell cultures would best ensure recovery of C. trachomatis. This would not be feasible for this laboratory, so samples must be stored at 4°C or -70°C. Several types of transport media were evaluated to determine which would be the most effective for storage and transport. 2SP and SPG are specialized media used for transport of C. trachomatis, and Hanks' BSS and T-soy broth were included in this study because they are used for transport of viral specimens, and can be found routinely in many clinical laboratories. Dimethyl sulfoxide (DMSO) and glycerol are cryoprotective agents used in preserving cells at -60 to -70°C; they were added to 2SP to determine if these agents aided in the survival of C. trachomatis after freezing.

When evaluating the different media for survival of the organism it was noted that the recovery of C. trachomatis was different for each inoculum-suspending medium tested. Since these samples were placed on the cells immediately after the dilutions were prepared, the effects must be due to the effects of the constituents of the media on adsorption and/or

phagocytosis, rather than the organism's loss of viability. The pH or cation concentration of the medium may be a factor in the numbers formed, since the interaction of the chlamydial EB with the host cell is electrostatic in nature (12,13).

The most pronounced effects were seen with DMSO and glycerol supplements, where only 15% of the inclusions found in 2SP were recovered in 2SP containing these additives. The addition of glycerol increased the viscosity of 2SP, and this may have physically inhibited contact of the organism with the host cell. The DMSO supplement increased the pH of 2SP from 7.2 to 7.5 which may have affected the attachment of the chlamydial EB. However, the differences in pH between the other four media were small (7.3 ± 0.1), and the recovery of C. trachomatis was still affected. The superiority of 2SP as an inoculum-suspending medium has been attributed to its low salt content, and high levels of sodium chloride have been shown to inhibit binding of C. psittaci to L cells (13,67). This could explain the differences shown since Hanks' BSS and T-soy broth have relatively high concentrations of sodium chloride, and less inclusions were recovered with these media than 2SP or SPG. The effects of inoculum-suspending medium on the efficiency of C. trachomatis infection of McCoy cells have been reported in the absence of centrifugation (67), but this is the first report of these differences when chlamydial infection was aided by centrifugation. It is possible that these differences are negated when higher centrifugal forces ($> 900 \times g$) are used, since centrifugation can overcome the factors needed for attachment of chlamydiae to host cells (69). However, centrifugation was performed at $900 \times g$ throughout this study, and 2SP consistently yielded higher numbers of IFU when tested

at this speed.

2SP medium appeared superior to the other media tested for storage of C. trachomatis at 4° and -70°C also. Although the recovery of the organism on SPG was essentially equivalent to the recovery in 2SP after one freeze-thaw cycle, the overall effectiveness of SPG for storage at 4°C, and as an inoculum-suspending medium was not equal to 2SP medium. The addition of DMSO and glycerol may have improved the quality of 2SP for maintaining the viability of the organism at -70°C, but these agents reduced the effectiveness of 2SP as an inoculum-suspending medium. Since none of the media tested showed significant improvements over 2SP for storage of C. trachomatis at 4°C and -70°C, further comparisons were not performed.

In 2SP medium, more infectivity of C. trachomatis is reported to be lost by freezing at -70°C, than by keeping the specimens at 4°C for 24 to 48 hours (42,56). The results of this study were consistent with this, as the viability of the organism decreased approximately 20% per day at 4°C, and an average of 55% after one freeze-thaw cycle. Samples stored in 2SP at -70°C did not lose significant numbers of chlamydiae over a prolonged period of time, which is advantageous for batching of samples to be tested.

The 2SP medium was provided for the collection of clinical specimens with the following recommendation: if the specimen could not be transported within 24 hours, it should be kept frozen for transport and storage (56). The results obtained with the clinical specimens reflect the importance of this recommendation for isolation of C. trachomatis. Ten of the 139 samples tested were held at 4°C and processed within 24 hours of collection. Two (20%) of these samples were positive for Chlamydia.

Fifteen samples were received that had been frozen, but thawed during transport. These samples could not be tested immediately, and were frozen at -70°C . C. trachomatis was not detected in any of these specimens. Ten Chlamydia-positive samples found in comparing iodine and PAP stains were frozen at -70°C and tested a second time with iodine and the IFA assay, but only eight samples remained positive. Each of the two negative specimens had less than five IFU on the first staining. Again, this is a minimal number of samples, but it appears that transport and storage of specimens can affect the isolation of C. trachomatis, especially in samples containing low numbers of infective particles.

Low numbers of chlamydiae in clinical specimens were not as readily detectable with the immunoperoxidase (PAP) assay. The differences in sensitivity seen between iodine staining and the PAP assay were in samples containing less than 20 IFU per coverslip with the iodine stain. At higher levels of infectivity (> 20 IFU per coverslip), iodine staining, the PAP assay, and IFA assay appeared comparable for the detection of C. trachomatis.

The coverslips stained with the PAP and IFA methods were difficult to examine and interpret because of the problems with nonspecific staining. Precipitated stain, cellular debris, and epithelial cells often resembled intracellular inclusions, so scanning of monolayers was prolonged by using higher power magnifications to prevent false positive determinations. When chlamydial inclusions were present, they were usually very distinct with both stains, so nonspecific staining does not account completely for the differences in sensitivity found with iodine staining and the PAP assay. Only a limited number of samples

were tested with the IFA technique, and it is difficult to evaluate the overall effectiveness of this method. However, the lack of sensitivity seen with the PAP method may apply to the IFA assay as well, since the antibody reagents were purchased from the same company. A monoclonal antibody to a group-specific antigen common to both C. trachomatis and C. psittaci was employed in both assays. In studies where monoclonal antibody techniques were shown to be superior to iodine and Giemsa stains, Stamm et al (59) and Stephens et al (60) used monoclonal antibodies to species-specific antigens of C. trachomatis. This may indicate that the antibodies to the group-specific antigen are more broadly-reactive, and less sensitive for the detection of C. trachomatis. The opposite effect may be that monoclonal antibodies to a single antigenic determinant are too specific and antibody interaction may be limited by the expression of the antigen (70). Monoclonal antibodies to group-specific antigens have been shown to react preferentially with RB forms of both C. trachomatis and C. psittaci, whereas antibodies to species-specific antigens react equivalently to both the RB and EB forms (58). These differences in specificity between monoclonal antibodies to group and species-specific antigens could contribute to the effectiveness of an immunodiagnostic assay.

Theoretically, the IFA and PAP assays would be superior to iodine staining since antibody methods detect all antigenically intact inclusions of C. trachomatis. Iodine stains only those inclusions containing glycogen (which will vary depending upon the stage of the growth cycle) (71). The results of this study were unexpected, but both the IFA and PAP assays require some expertise in reading and interpretation of stained monolayers; this may have been lacking with the number of

samples tested. Even if the PAP assay had been shown to be more effective than iodine staining, a distinct disadvantage of this method was the time involved in staining the coverslips (three hours). Iodine staining of cell monolayers could be performed in ten minutes, and each coverslip could be screened within one to two minutes because there were no problems with nonspecific or background staining.

Another disadvantage of the PAP assay was the cost of the reagents. The cost per specimen with the PAP assay will range from \$4 to \$8, depending on whether the coverslips were stained on the serological glass slide or in the culture vials, respectively. Both methods were performed in this study with no differences seen in the quality of staining or the time involved. The cost of reagents for the IFA assay would be comparable to the cost of the PAP reagents. However, iodine stain can be prepared in the laboratory for a cost per specimen of less than 5 cents. It would appear that iodine staining was the least expensive, and the most practical and effective method for detection of C. trachomatis tested in this study.

The 139 clinical specimens were obtained from female patients in obstetrics and gynecology clinics. The isolation rate of C. trachomatis from these specimens was 9% with iodine staining of McCoy cell cultures. The recovery rate of C. trachomatis from cervix-vaginal specimens tested at Mayo Clinic from 1974 to 1981 was 7.3% (66). Again these prevalence rates give some assurance to the sensitivity of the tissue culture isolation method used in this laboratory. Further studies with clinical specimens tested in conjunction with a reference laboratory would better establish the overall effectiveness of the method.

Since C. trachomatis is frequently found concomitantly with

Neisseria gonorrhoeae in females (28), a retrospective analysis of the 139 patients tested for C. trachomatis revealed that of 132 specimens tested for gonorrhea, N. gonorrhoeae was isolated from only one patient. C. trachomatis was not isolated for this particular patient. Although the rates of dual infection were nonexistent in this study, these results indicate the prevalence of C. trachomatis, and the relevance of clinical testing and treatment for chlamydial infections.

The cost effectiveness of the chlamydiae isolation procedure would be dependent upon the number of specimens received for testing. Judging from the numbers received for this study over a four-month period, diagnostic testing would be feasible if specimens were batched (five or more specimens) and tested once a week. This would mean a turn-around time for results of 1 to 2 weeks, which could limit the value of the test procedure. However, it should be considered that the specimens tested were from a select group of patients, and the number of specimens submitted may be higher when male and pediatric populations from all health facilities are included. The results of this study suggest a need for diagnostic testing, since the isolation rates obtained are comparable to isolation rates reported from laboratories routinely testing for C. trachomatis. The feasibility of providing a diagnostic isolation procedure for C. trachomatis would depend upon the demand for this test in our area. Before diagnostic testing could be performed, one problem which would need to be overcome is the transport of clinical specimens. The health care personnel would have to be made more aware of the necessity of shipping specimens so they remained at 4°C or frozen during transport to this laboratory.

In conclusion, combining the use of McCoy cell monolayer cultures

with iodine staining proved to be the most effective and practical method for isolation of C. trachomatis in this laboratory. McCoy cell monolayer cultures were more sensitive than McCoy cell suspension cultures or L-929 cells for the recovery of chlamydial inclusions. Iodine staining was more efficient than immunoperoxidase and immunofluorescence assays for the detection of C. trachomatis in McCoy cell monolayer cultures. 2SP medium was shown to be superior to the other media tested as an inoculum-suspending medium and for storage of C. trachomatis at 4°C and -70°C.

SUMMARY

McCoy cell monolayer cultures were compared to L-929 cells, and McCoy cell suspension cultures to determine the most effective tissue culture method for isolation of C. trachomatis. In an initial study, more chlamydial inclusions were detected with McCoy cell monolayer cultures than with L-929 cell monolayer cultures. McCoy cell suspension cultures were used to eliminate the need for preformed monolayers of cells, and recovery rates of chlamydiae in suspended cells were high in three of five trials when compared to monolayer cultures. Subsequent studies revealed suspended McCoy cells to be more susceptible to adverse effects from bacterial contamination, toxic products, and temperature variations than McCoy cell monolayer cultures. Overall, McCoy cell monolayers were more stable and more sensitive for isolating C. trachomatis, so cell monolayers were used to evaluate the effects of transport media, and of staining techniques used for tissue culture isolation of the organism from clinical specimens.

Growth of C. trachomatis in tissue culture cells is dependent upon centrifugation of the chlamydial inoculum to enhance contact of the organism with the host cell. Centrifugation was performed initially at 500 x g at 36°C for one hour. Increasing the centrifugal force increased temperatures within the centrifuge. Recovery rates of C. trachomatis after centrifugation at 500 x g at 36°C for one hour were nearly double the rates obtained with centrifugation performed at 900 x g at room temperature for one hour. However, temperature variations within the centrifuge could not be controlled, so centrifugation was

carried out at 900 x g at room temperature for the duration of this study.

Six media were evaluated as inoculum-suspending media, and for their ability to maintain the viability of C. trachomatis at 4°C and -70°C. The media tested included 2SP, SPG, Hanks' balanced salt solution, T-soy broth, 2SP containing 10% dimethyl sulfoxide, and 2SP containing 10% glycerol. 2SP was superior to the other media tested as an inoculum-suspending medium, and for the storage of C. trachomatis at 4°C. 2SP and SPG were comparable for maintaining the viability of the organism after one freeze-thaw cycle at -70°C, but less inclusions were recovered from SPG before and after storage at 4°C. 2SP was used for transport of clinical specimens procured for this study. The viability of C. trachomatis in 2SP medium decreased by approximately 20% per day at 4°C, and an average of 55% after one freeze-thaw cycle at -70°C. The viability of the organism was not decreased by prolonged storage at -70°C in 2SP medium.

The effectiveness of iodine staining, immunoperoxidase (PAP) and immunofluorescence (IFA) assays for the detection of C. trachomatis in McCoy cells was evaluated by testing clinical samples procured from four area health facilities. From 139 patient samples tested, 12 (9%) were positive for C. trachomatis with iodine staining, whereas only 7 (5%) were positive for chlamydiae with the PAP assay. Inclusions were the most difficult to detect with the PAP assay when less than 20 inclusions per coverslip culture were detected with iodine staining. Ten of the twelve positive samples were stored at -70°C and tested a second time with iodine stain and the IFA assay. Only eight samples were positive for chlamydiae with iodine stain, and

seven were positive with the IFA assay. Iodine staining of cell cultures was more sensitive, less expensive and less time-consuming than the PAP or IFA assays.

The isolation rates of C. trachomatis from clinical specimens using McCoy cell monolayer cultures and iodine staining appear comparable to recovery rates obtained from laboratories routinely testing for chlamydiae. Although further studies would be needed to determine the overall effectiveness of this method, diagnostic isolation of C. trachomatis would be feasible for this laboratory if sufficient numbers of samples were received.

Appendix A - Key to Abbreviations

CF: complement-fixation test

CGM: Chlamydia growth medium

CMF-PBS: calcium-magnesium-free phosphate buffered saline

DEAE-dextran: diethylaminoethyl-dextran

DMSO: dimethyl sulfoxide

EB: elementary body or infectious particle

EBSS: Earle's balanced salt solution

FBS: fetal bovine serum

Hanks' BSS: Hanks' balanced salt solution

IFA: immunofluorescence assay (Immulok, Inc., Carpinteria, CA)

IFU: inclusion-forming units

IuDR: 5-iodo-2'-deoxyuridine

LGV: lymphogranuloma venereum

MEME: Eagle's minimum essential medium containing Earle's balanced salt solution and L-glutamine

M199: medium 199

Micro-IF: microimmunofluorescence test

NGU: nongonococcal urethritis

PAP: immunoperoxidase assay (Immulok, Inc., Carpinteria, CA)

PGU: postgonococcal urethritis

PID: pelvic inflammatory disease

RB: reticulate or initial body

2SP: 0.2 M sucrose in 0.02 M phosphate buffer

4SP: 0.4 M sucrose in 0.02 M phosphate buffer

SPG: sucrose-phosphate-gultamate medium

TRIC: trachoma-inclusion conjunctivitis strains of C. trachomatis

T-soy broth: tryptic soy broth

Appendix B - Chlamydial Media and Stains

Sucrose-phosphate-glutamate medium (SPG) (31)

Component	Amount
Sucrose	75.00 gm
KH_2HPO_4	0.52 gm
Na_2HPO_4	1.22 gm
Glutamic acid	0.72 gm
Distilled water to	1000 ml

The pH was adjusted to 7.4 with 1.0 N NaOH. The medium was sterilized by autoclaving for 15 minutes at 15 pounds pressure (121°C).

Jone's Iodine Solution (64)

Component	Amount
Potassium iodide	20.0 gm
Iodine crystals	20.0 gm
95% ethanol	200.0 ml
Distilled water	200.0 ml

The iodine was added to the alcohol, and then the potassium iodide was dissolved in this solution. Water was added to make final volume. The solution was filtered twice through Whatman #1 filter paper, and stored in brown colored bottles at room temperature. Expiration date: 6 months.

Jone's Iodine-Glycerin (64)

Equal amounts of Jone's iodine solution and glycerin were mixed and stored in a dark bottle at room temperature. Expiration date: 6 months.

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