COLLECTION, HANDLING & SHIPMENT

OF MICROBIOLOGICAL SPECIMENS

U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE

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OF MICROBIOLOGICAL SPECIMENS

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FOREWORD

A reliable laboratory report as an aid in the diagnosis of disease depends upon the care and thought used in the collection, handling, and transport of specimens. Too often physicians and other medical or public health personnel are only generally familiar with the problems and procedures involved in obtaining and submitting material for laboratory examination.

The objective of this manual is to present procedures which, in the opinion of the staff of the National Communicable Disease Center—who authored the various sections—have been found to be practical and productive. Other methods may be equally satisfactory, however, and may, at times, be substituted for those described. In any case, whenever laboratory examinations are to be carried out in local or State laboratories, methods acceptable to these laboratories should be used.

It is of utmost importance that the purpose of a procedure for handling a specimen be kept in mind. Thus, for example, the safest and most expeditious method of transporting specimens to a diagnostic laboratory may be by automobile rather than by mail or express, thus obviating the need for elaborate packaging. In contrast, forwarding specimens to a reference library by mail requires procedures which insure viability of the infectious agent and provide maximum protection to those handling the shipment in transit.

Use of trade names is for identification only and does not constitute endorsement by the Public Health Service or the U. S. Department of Health, Education, and Welfare.

CONTENTS

CONTENTS—Continued

TABLES

FIGURES

APPENDICES

Laboratory Services at the National Communicable Disease Center

The laboratories of the National Communicable Disease Center (NCDC) serve as a national reference or consultative facility and accept specimens from only the State public health laboratories and Public Health Service facilities. Specimens which cannot be examined locally should be sent to the State laboratory where they may be processed or, if the requested service is not available at the State level, the State laboratory director may then forward either the original specimen or the pure culture to the NCDC. Certain services available at NCDC are of value in epidemic situations only, and submission of single cultures from isolated cases would serve no useful purpose. Requests for serotyping of Group A streptococci and phage typing of *Salmonella, Shigella,* or staphylococci from such cases serve to illustrate the point. Acceptance of diagnostic specimens from private physicians or institutions and local health departments is not authorized.

The volume of specimens submitted for virological examination has increased to the point that it is impossible for NCDC to examine routine specimens and, at the same time, provide the support needed for development of virological facilities within the State laboratories and assist the States in the solution of their epidemiologic problems. Because assistance to the States in the above matters is of major importance and a basic responsibility of NCDC, the resources of NCDC virology laboratories must be directed to these ends. Therefore, the Laboratory Program *limits* its acceptance to specimens having epidemiologic implications.

Since virology is still a rapidly developing science, diagnostic problems may occasionally arise which require assistance from the NCDC laboratories even though the case has no immediate clear-cut epidemiologic implication. In such instances, the State laboratory director should consult with the Chief of the Virus Reference Unit at the NCDC.

From the viewpoint of the practicing physician, this policy should work no hardship since most virological diagnosis is retrospective and of little or no value in the treatment of the individual patient.

Procedures for Collection, Preparation, and Shipment of Microbiological Specimens

GENERAL INSTRUCTIONS

1. **Basic Principles.** In obtaining and submitting diagnostic specimens, basic principles should be observed. Principles applicable to specimens submitted for isolation and identification of the etiologic agent include:
   a. Select appropriate specimens.
   b. Obtain specimens as early in the illness as possible.
   c. Identify the specimen properly.
   d. Request specific test(s) desired or indicate etiologic agent suspected.
   e. Protect viability of organisms during transit.
   Other principles applicable to specimens submitted for serological examination are:
   a. Collect blood and serum samples in sterile tubes and maintain sterility in handling. Use leakproof stoppers on all tubes.
   b. Collect paired specimens at appropriate intervals because, with a few exceptions, they are desirable and because, in suspected viral infections, they are mandatory. Since a rise in antibody titer is a more reliable diagnostic sign than the mere presence of antibodies, the first specimen should be collected as early after onset as possible and the second, usually two or three weeks later.
   c. Protect whole blood from freezing under any circumstances.
   In general, specimens submitted as smears for examination should:
   a. Be allowed to dry completely.
   b. Be packed properly to prevent breakage.

2. **Infectious Diagnostic Specimens.** Rapid transport of infectious diagnostic specimens is of vital importance in communicable disease control. Fortunately, no regulations hamper such movement as long as one follows the rigid Postal Regulations which pertain to the *preparation* of the specimen for shipment.
   The NCDC defines infectious diagnostic specimens as:

   a. All specimens of human or animal excreta, secreta, tissue, tissue fluids, or hair which contain or are suspected of containing the live causative agent of a human disease or an animal disease transmissible to man, and which are shipped or mailed to a diagnostic or research laboratory for isolation and identification of the etiological agent.
   b. Pure cultures or concentrated isolates or vectors of etiological agents shipped from the isolating or collecting laboratory to a specialty laboratory for identification and typing, or further research, or both.
   c. Pure cultures of known etiological agents which are used as reference cultures or as antigens in diagnostic laboratory procedures.

3. **Identification of Specimens.** Identify individual specimen tubes or containers by encircling them with *typed or penciled legends on adhesive tape.* Give patient's name, type of specimen, and date of collection. This is particularly important with clear fluids and paired sera. *Ink, ball-point pen, wax, or indelible pencil should not be used* because writing done with them becomes illegible. *Include with the shipment* a legible copy of a list of the specimens giving identifying name or number, date obtained, and tests desired.

4. **Packaging of Specimens.** Proper packaging not only protects the specimen in transit but also the personnel handling it. Protection is especially important in the case of breakage.

   Never Mail Viable Specimens in Petri Plates. Never Enclose Dry Ice in Hermetically Sealed Containers.

   A safe packaging procedure which complies with Public Health Service Regulations and the requirements of the Universal Postal Union follows:
   a. Enclose the specimen in a bottle or tube of thick glass sealed with a rubber stopper or

2

paraffin-treated cork. Bottles are preferred because of their greater shock resistance, but heavy-walled tubes are acceptable if properly packed. Closure by fusion is also acceptable.

b. Place the glass container in an airtight and watertight tin to absorb any leakage, pack absorbent cotton, vermiculite, sawdust, or other suitable absorbent material around the glass container.

c. Pack the can in a cardboard container with crumpled newspaper or other shock-resisting insulating material and wrap for shipment.

d. Individual tubes must be shipped in containers providing sufficient space for shock-absorbing material all around the tube. If several tubes are packed in the same can, wrap them individually in absorbent material such as soft paper or cloth to provide adequate cushioning between the tubes.

e. SCREW-CAP TUBES ARE NOT RECOMMENDED for blood, serum, or other fluid specimens because leakage frequently occurs, particularly when outside pressure decreases during air transportation. Screw-cap tubes are acceptable for agar or similar cultures.

f. Bottles or tubes for specimens should be of hard glass and of 2 ml. or larger capacity.

g. Screw caps should have a resilient, sealing gasket or insert and should be secured by tape.

h. When screw-capped *jars* are used for shipping larger specimens, the resilient cap lining should achieve airtight and fluid-tight closure, and the cork or cap should be secured in place with a metal collar or adhesive tape. Gas-forming cultures of yeast are an exception to this rule.

i. Regular #3 household cans or pressure-sealed paint cans are useful for shipment of bottles or multiple specimens. Household cans should be sealed by roll crimping the lid with a home-canning device. Pressure-sealed paint cans have the advantage of not requiring a crimping device. The large sized paint cans are practical for large quantity shipments and may be used as outer containers, as required by the Convention of the Universal Postal Union.

5. **Shipment of Specimens.**
   a. Mark shipments with "Perishable," "Packed in Dry Ice," "Refrigerated Biologic Material," "Fragile," or some other suitable designation. Standard labels should be used if available.

b. For long distances, ship all specimens by air mail or air express. Air freight should not be used when speed is essential. If possible, assure that the shipment is given priority over nonperishable items.

c. Specimens submitted to the laboratory should be accompanied by an appropriate report form giving name and age of patient, source of specimen, disease suspected, brief statement of clinical symptoms, and, in the case of cultures, tentative identication.

d. Delay may be avoided by addressing shipments to the laboratory unit involved; for example, "Virus Reference Unit," "Enteric Bacteriology Unit," "Venereal Disease Research Laboratory," etc.

e. Shipment of specimens should be timed so that they will not arrive at the laboratory on or just before a weekend or holiday; this will help avoid possible deterioration.

f. In some localities, surface mail, bus or railway express may be faster than air transport; but, in any case, the most rapid method should be used.

6. **Criminal Statute (18 USC 1716) Pertaining to Shippers of Diagnostic Materials.** This statute is of interest to all shippers of diagnostic materials whether the materials are potentially pathogenic or not. Even if spillage occurs from non-pathogenic materials but injures or damages mail, equipment, or personnel, the shipper may face prosecution even though there is no question of hazard from an infectious agent. The value of meticulous packaging, with sufficient absorbent material around the specimen to prevent fluid leakage, extends well beyond the major concern of preventing accidental infection.

BACTERIOLOGICAL, MYCOLOGICAL, AND PARASITOLOGICAL SPECIMENS

For the purpose of this manual, the significant difference between primary specimens and cultured bacteria and fungi is that primary specimens are usually shipped intrastate only, whereas most shipments to NCDC or other out-of-State reference laboratories consist of pure cultures. The same general rules and principles covering handling and shipment of pure

cultures also apply to specimens obtained from the patient or any other source.

1. *Bacterial Specimens.* Agar slant or stab cultures, using freshly prepared media free of excess moisture, are most practical. Stab cultures are best for anaerobes. If the bacteria have low shipping tolerance (as does *Neisseria,* for example), fresh and relatively heavy growths on either blood agar or brain heart infusion slant, moistened with a drop of blood, or a chocolate agar slant should be forwarded. The tube should be closed with either a rubber stopper or paraffin-treated cork secured in place with adhesive tape. A screw-cap tube is also acceptable, provided the cap is wrapped and sealed with tape. The tape should be applied in the direction of the threads to avoid loosening the cap. Viable cultures should NEVER be shipped in Petri plates.

2. *Mycological Specimens.* Yeast cultures which develop gas are the single exception to the rule that specimens should be placed in tubes or bottles sealed with rubber stoppers or paraffin-treated corks. In this instance, the culture tube should be plugged with a dry, nonabsorbent, tight fitting cotton plug, long enough to extend into the tube about one inch. This plug should be held in place with adhesive tape in such a manner that gases will escape, but the plug will not work loose. Slant cultures on firm agar are preferred over stab cultures. Other fungal cultures must be shipped only in sealed tubes or bottles.

3. *Parasitological Specimens.* Stool specimens to be examined for intestinal protozoa should be submited in two parts: one in polyvinyl alcohol (PVA) fixative solution, the other in formalin. In suspected cases of filariasis or trypanosomiasis, whole blood should be heparinized and sealed in a tube as for bacterial specimens. It may be necessary to submit samples of arthropod ectoparasites in two portions: one for identification, the other for viral or rickettsial isolation.

Mosquitoes and flies should be sent to the laboratory unmounted, preferably in pill boxes between layers of cleansing tissue but *never between layers of cotton.* If viral or rickettsial isolation is to be attempted, the arthropod specimens must be collected alive, sealed in ampules, and stored and shipped on dry ice. Cyanide or chloroform jars must not be used because viral and rickettsial agents are inactivated by these materials.

NOTES: Special precaution in regard to safe packaging is required when shipping the etiological agents of especially dangerous diseases such as plague, cholera, anthrax, tularemia, and coccidioidomycosis. Label tubes or bottles containing such organisms with precautionary labels and enclose them in double metal containers.

In some instances, reference cultures of bacteria are maintained either in the sand desiccated or lyophilized state. Such cultures are shipped in the same manner as agar cultures.

VIRAL AND RICKETTSIAL SPECIMENS

If isolation of a virus is to be attempted, the source of the specimen should be carefully selected, and the specimen should be obtained during the early, acute, febrile phase of illness.

Depending on the circumstances, the material for virus isolation may be either nasal or throat washings, sputum, feces, cerebrospinal fluid, scrapings, aspirations from lesions, or tissues from autopsies. Blood, spinal fluid, and tissue should be handled aseptically. Isolations of rickettsia may be obtained from whole blood. Unless specimens for virus isolation can be delivered to a laboratory within three hours, it is mandatory that they be frozen and kept frozen during shipment. An exception is a specimen suspected of containing respiratory syncytial virus. If freezing is impossible, tissue specimens may be placed in buffered glycerin for transport; but the results may be equivocal. This procedure is impossible with body fluids.

Possible delays in transit necessitate the use of sufficient dry ice in the shipping container to insure freezing for 48 hours in excess of the normal transit time.

Since changes in atmospheric pressure during air shipment may force stoppers from tubes, resulting in loss of the specimen, only tight fitting corks or soft rubber stoppers should be used; and, in any case, the cork should be anchored with adhesive tape. When a highly infectious disease such as psittacosis or smallpox is suspected, several layers of gauze soaked in 4% formalin should be wrapped around the tube or bottle containing the specimen before it is placed in a metal, leakproof shipping case. Substitution of 10% cresol solution for the formalin is permissible. Specimens for serologic tests indicative of viral in-

fection must be taken aseptically, and 10 to 15 ml. of blood should be drawn. Paired specimens are essential to diagnosis as a rise in specific antibody titer in the course of illness and convalescence is the only definitive serologic evidence of current infection. The first specimen should be taken during the acute phase of illness and the second, two to four weeks later, although the optimum times for the collection of specimens vary with the disease. After the blood has clotted and the serum has separated, remove the serum promptly and aseptically, and immediately refrigerate or freeze it. Do not add a preservative, as this would destroy the serum's usefulness.

MISCELLANEOUS SPECIMENS

1. If bacterial or fungal serology only is desired, draw 10 ml. of blood. This amount will yield sufficient serum because of the smaller number of antigens used in the tests.

2. Sera intended for only bacterial tests or fungal serology may be preserved by adding merthiolate to make a final concentration of 1:10,000 (0.01 ml. of 1% merthiolate to each ml. of serum).

3. When an infection has been treated with an effective antibiotic, antibody development may be suppressed or delayed, and, in such cases, a third blood specimen taken late in convalescence may be helpful.

4. A presumptive diagnosis on the basis of a high serologic titer in a single convalescent blood specimen may be possible. This procedure is most often useful in confirming an epidemic situation where paired specimens are also available from a significant number of individuals. This is also true in cases of rare diseases such as glanders, typhus (not Brill's disease), or yellow fever, in which previous exposure is only a remote possibility.

Directions for the Collection of Specimens for the Laboratory Diagnosis of Certain Bacterial, Mycotic, Parasitic, Arthropod-Borne, Viral and Rickettsial Diseases

In any epidemic situation involving the collection of specimens for laboratory examination, the epidemiologist should consult the director of the laboratory where the work is to be done to make sure that: (a) the tests to be requested are available, (b) the culture media and reagents required will be ready when needed, and (c) the collection and submission of specimens is scheduled at a rate that will not overtax the facilities.

BACTERIAL DISEASES

1. *Anaerobes* (diseases due to). The major areas of public health interest in which anaerobic bacteria are implicated are: 1) wound infection, 2) food poisoning outbreaks due to *Clostridium botulinum,* 3) food poisoning due to *Clostridium perfringens,* 4) tetanus, and 5) infections with non-sporeforming anaerobic bacteria. Specimens may be as diverse as food, blood, tissue, cerebrospinal fluid, or materials from wounds, abscesses, and serous cavities. Culture and toxin testing of food samples should be attempted in a local laboratory only if the personnel are experienced in anaerobic techniques. Otherwise, the specimen should be forwarded to the nearest competent reference laboratory. Certainly, however, a smear of the original food specimen should be made for Gram staining since study of this smear will give the laboratorian a census of the relative numbers and different kinds of bacteria present. Such information may be important in concluding whether a presumptively significant organism was recovered during culture.

a. **Food specimens for isolation of Cl. botulinum and/or toxin**
Specimens should be collected and maintained at the same temperature as in the natural state for transport to the laboratory or shipment to a reference laboratory. Laboratory testing consists of detection and typing of *Cl. botulinum* toxin in the food as well as isolation and identification of the causative organism.

b. **Food specimens for enumeration of Cl. perfringens**
Quantitation of *Cl. perfringens* from food should be performed in a local laboratory as soon as possible after the food is collected. If the material *must* be shipped, it should be refrigerated at 4°C. and maintained at this temperature during transport. At 4°C., some organisms will die, thus causing lower counts. This fact should be kept in mind in interpreting counts. Freezing of specimens drastically lowers counts, and shipment without refrigeration permits multiplication of the organisms, thus producing unusually high counts.

c. **Specimens from human sources**
Clinical material must be cultured immediately after collection since some anaerobes die very rapidly upon exposure to oxygen in a nonprotective environment. Where possible, aspirated fluids are preferable to swabs. Never allow material on swabs to dry out. If a specimen cannot be cultured immediately, it is helpful to place the material in a medium containing a reducing

agent, such as fresh thioglycollate broth, at room temperature for a period not exceeding two hours.

A Gram stained smear should be prepared on all specimens, and each type of organism seen in smears should be isolated in pure culture at the local laboratory. Pure culture isolates can be shipped to a reference laboratory for final identification. Thioglycollate medium, enriched with 10% normal rabbit serum, is preferable for isolation of the non-sporeforming anaerobes, and chopped meat medium (Appendix II) is preferable for the sporeforming anaerobes. It is important to plate specimen material directly on fresh blood agar plates since some fastidious anaerobes may be overgrown by other organisms in broth medium. Broth cultures are incubated 24 hours, preferably in an anaerobe jar; Gram stains are prepared, and a second set of blood agar plates is inoculated. Blood agar plates should be incubated in an anaerobe jar for a minimum of 48 hours. Isolated colonies should then be picked to chopped meat or thioglycollate medium for isolation of pure cultures.

For shipment of pure cultures to a reference laboratory, actively growing cultures in screw-cap tubes of chopped meat medium, thioglycollate medium, or semi-solid thioglycollate medium with 0.5% added agar can be used. Prior to shipment, a one-half inch column of sterile 5% agar should be layered over the culture to act as a seal, and the cap should be sealed with waterproof tape.

2. *Anthrax.* Anthrax is primarily an infectious disease of animals; however, man may be infected through contact with infected animals or animal products. The majority of cases are cutaneous infections, but pulmonary and intestinal infections may be seen. "Industrial anthrax" occurs in workers exposed to carpet wools, goat hair, or skins originating in areas where the disease is prevalent among animals. Laboratory diagnosis is made by smear examination, culture of the organism, animal inoculation, or fluorescent antibody technic.

Specimens for laboratory diagnosis

a. Smears may be made from sputum, blood, other fluids, or tissues, and then air dried and heat fixed.

b. Blood, vesicle fluid, and scrapings from the base of the lesion, regional lymph nodes, or other organs, taken in as clean a manner as feasible, may be cultured on plain or blood agar or inoculated into animals.

c. Tissue impression smears, sputum, other clinical material and cultures may be stained with fluorescent antibody for specific identification of the organism.

d. Aqueous extracts of soil samples, heat-treated to destroy vegetative cells, may be cultured or used for animal inoculation.

All types of examinations should be made in the nearest competent laboratory because delay in the shipment of fluids or tissues may result in contamination of the specimen and death of the organism.

3. *Brucellosis.* This disease is found primarily in goats, cattle, and swine. Man contracts the disease either by direct contact with diseased animals or through consumption of infected milk and milk products. Although the three organisms, *Brucella melitensis, Brucella abortus,* and *Brucella suis,* most often infect goats, cows, and pigs, they are not host specific, and all infect man as well. The disease occurs in acute, subacute, and chronic forms; and all three types are produced by any of the species of *Brucella.* Infection occurs most frequently by direct invasion of the organism through the intact intestinal mucosa; but stockyard workers, farmers, and veterinarians are often infected through the skin by direct contact with living or dead tissues of infected animals. Laboratory diagnosis is made by agglutination tests, animal inoculation, or recovery of the organism in cultures.

a. **Specimens for isolation of the agents**
Draw 10 to 15 ml. of blood directly into a "B-D" blood culture medium bottle and send it to a local laboratory for incubation. Due to the intermittent appearance of organisms in the blood stream, it may be desirable to take additional specimens at intervals of several hours or days, depending on the condition of the patient.

b. **Specimens for serologic tests**
Since an agglutination titer of about 1:100 or higher is believed indicative of brucellosis, successive blood specimens taken three to six weeks after onset of illness are considered sufficient for diagnostic purposes.

4. *Diphtheria*. Specimens for diphtheria should be collected from both the nose and throat. Two sterile swabs should be used for each person cultured, one swab to obtain a specimen from the throat lesions or tonsillar crypts and the other to collect materials from the nasopharynx. Swabs should be kept separate during transport to the laboratory.

Specimens for isolation of the agent
To obtain nasal and throat specimens, the swab should be introduced into the nares at a right angle to the plane of the face, inserted completely into the nasopharynx, and rotated over the pharyngeal surface near or in the tonsillar fossa. Because a nasal membrane is a particularly good source of organisms, the swab should be placed at the margin of the membrane, if present, and the specimen taken without excessive bleeding.

Preferably, the initial isolation of organisms suspected of being *Corynebacterium diphtheriae* should be carried out in a competent laboratory near the source of the specimens. If it will require more than two hours to get the specimens to the laboratory, they should be inoculated to Loeffler's slants and incubated overnight before transporting them. The nasopharyngeal and throat swabs should be rotated gently over the surface of separate slants of the medium. Care should be taken not to break the surface of the medium. The swab should then be placed in a separate tube to be sent to the laboratory with the slant.

On arrival in the laboratory the inital swab is inoculated to a Loeffler's slant, a tellurite agar plate, and a blood agar plate. If the specimen is received on Loeffler's medium, a smear is made for microscopic examination, and a representative sample of the growth is inoculated to tellurite and blood agar plates. The use of blood agar is necessary to demonstrate the presence of Group A streptococci, or strains of *C. diphtheriae* which may be inhibited on the tellurite medium.

In carrier surveys, both swabs may be streaked gently and repeatedly over the surface of a single slant. Be sure to rotate the swabs between the thumb and forefinger as the swabs are drawn back and forth over the medium. Streaking of tellurite plates with the initial swabs taken in carrier surveys is of little advantage. On occasion, inhibitory lots of Loeffler's medium have been encountered due to the presence of anti-biotics in the serum from which the medium was prepared. Precaution should be taken, therefore, to make sure that all lots of the medium to be used will support growth of *C. diphtheriae*. Diagnosis from smears made directly from the patient should never be attempted since many other organisms morphologically resembling *C. diphtheriae* occur in the normal or diseased throat.

5. *Enteric Infections.* In suspected cases of typhoid fever and salmonellosis, specimens submitted for culture include blood, feces, and urine. Rectal swabs may be obtained by using an ordinary cotton-tipped applicator. Blood serum for agglutination tests is not recommended because such tests provide little or no significant information. *Blood cultures and serologic tests are not done in shigellosis.* The multiplicity of fluids suggested as transport media for specimens to be cultured indicates that none is ideal nor markedly superior to others, and, therefore, isolation of the agent should be attempted in the nearest competent laboratory.

a. Typhoid fever
Specimens to be collected for isolation of *Salmonella typhi*
(1) Blood taken early in the course of illness is the specimen of choice, but the probability of recovery of the organism decreases rapidly after 7 to 10 days of illness. Ten to 15 ml. of blood should be drawn directly into a "B-D" blood culture medium bottle (the volume of broth should be at least 10 times the volume of blood) and should be examined for growth after 3, 5, 7, and 14 days' incubation. If the blood is not drawn directly into the culture medium, an anticoagulant should be added.
(2) Stool examination is accomplished by adding approximately two grams, or a portion the size of a small marble, of *formed stool* to one ounce of Sachs 30% glycerol in buffered physiological-saline in a two-ounce, screw-cap bottle or "alkaselzer" bottle. Shake the bottle vigorously to emulsify the specimen before shipment. If *the stool is liquid,* add 2 ml. of the specimen to the preservative in the bottle. Be sure to include in the specimen any bits of mucosa or mucus present in the stool.

Before shipment of the specimen, make sure there is no leakage from the bottle. Amies' modification of Stuart's transport medium may be used.

(3) A "midstream" specimen of urine is collected as cleanly as possible, after cleansing the genitalia with soap and water and drying the area. Place the specimen in a tightly closed container for immediate delivery to the laboratory. In typhoid fever, stool cultures are more often positive than urine cultures.

(4) Specimens of food suspected of containing *Salmonella typhi* should be placed in ice cream cartons or other suitable containers and immediately refrigerated. *During transport to the laboratory, refrigeration must be maintained.*

b. Salmonellosis

Food poisoning may be caused by many members of the *Salmonella* group. Preliminary epidemiological investigation should reduce to a minimum the number of suspected foods likely to be responsible for any given outbreak, and indiscriminate collection of samples is unwarranted. Such inquiries will indicate the food consumed in common by those made ill, and, although such evidence is not infallible, it is at least presumptive.

Specimens to be collected for isolation of the agent

(1) If food from sealed containers is suspect, unopened containers of the same production lot should be submitted for testing. Also, representative samples of the suspected food should be transferred to a sterile sample bottle or ice cream carton and refrigerated during transit to the laboratory.

(2) Blood specimens for culture from patients in food poisoning are of limited value.

Blood cultures may be of value in the severe enteric, typhoidal, and septicemic types of salmonellosis. In such cases 10 to 15 ml. of blood may be drawn into a tube containing an anticoagulant or into a bottle of blood culture medium, as for typhoid fever.

(3) Fecal specimens, if obtained early dur-

ing the acute stage of the disease, are the specimen of choice and should be collected as for typhoid fever. If the specimens are to be mailed to the laboratory, they should be handled in the same manner as fecal specimens to be examined for *S. typhi*.

(4) Serologic studies on the sera of patients are not indicated since the results may be equivocal and impossible to interpret.

c. Shigellosis

Numerous members of the dysentery group of organisms are responsible for enteric disease. Their isolation is not difficult, and identification is accomplished by biochemical and serologic studies.

Specimens to be collected for isolation of the agent

(1) Blood specimens should not be submitted for cultural examination in suspected cases of shigellosis.

(2) Fecal specimens should be submitted for attempted isolation of the infecting organism at any stage of illness, but specimens will yield more successful isolations if they are obtained in the acute phase of the disease. Examination of a series of fecal specimens is important because the appearance of the organism in the stool may be intermittent. The specimen should be handled as one for typhoid fever.

(3) Rectal swab specimens are sometimes the most convenient specimens from which to attempt recovery of dysentery bacilli. This is especially true when examining inmates of institutions, hospitalized patients, or infants and children. Preferably, culture plates should be inoculated immediately after the specimen is taken, but the plates must be hand-carried to the laboratory for incubation; otherwise, the specimens should be transported to the laboratory as promptly as possible by placing the swab in Amies' modification of Stuart's transport medium.

d. Enteropathogenic *Escherichia coli*

A variety of agents may cause the diarrheal diseases of the newborn and infants. Thus, epidemics and sporadic cases of "summer" diarrhea, infantile enteritis, or diarrhea of

the newborn may be caused by *Salmonella, Shigella,* or viruses. Many otherwise unexplained epidemics of infantile diarrheal disease in which certain serotypes of *Escherichia coli* were recovered have, however, occurred. For these particular serotypes, the term enteropathogenic *E. coli,* or EEC, is commonly used. In order to control the spread of infantile diarrhea, the causative organism must be accurately and rapidly identified.

Earlier attempts at differentiation of the enteropathogenic coli strains were unsuccessful because only biochemical methods were employed and, as is now known, different *E. coli* serotypes produce identical biochemical reactions. These biochemically similar organisms fall naturally into certain groups, and serologic methods must be used to determine the serotypes within each biochemical group.

The determination of *E. coli* serotypes is dependent on the determination of the "O," "B," and "H" antigens of the bacterium. The *E. coli* responsible for the disease can be distinguished from the *E. coli* constituting the normal intestinal flora only by use of specific antisera.

Specimens to be collected for isolation of the agent

Fecal material should be collected either as a stool or on a rectal swab before antibiotic therapy is begun. The stool specimen or rectal swab should be placed in a screwcap vial containing 30% buffered glycerin-saline solution, or (if there will be more than a two-hour delay before it can be planted in the laboratory) on Amies' modification of Stuart's transport medium.

Specimens for enteropathogenic *E. coli* diagnosis by FA technic.

Specimens preserved with any fluids containing glycerol are not satisfactory for FA staining. A separate buffered swab (as supplied with Amies' transport medium) should be sent to the laboratory in a separate tube containing no preservative or medium.

6. *Gonorrhea.* Difficulties in the laboratory diagnosis of gonorrhea are increased when the clinician fails to exercise care in securing suitable exudates for examination. This is especially true in chronic gonorrhea of women and in "test of cure" where only minimal numbers of gonococci may be present in the exudates obtained from endocervical, Skene's, and Bartholin's glands. The exudate is taken with sterile cotton-tipped applicator sticks; the cotton tip should be small enough to enter easily the urethra and cervical os. Sometimes a platinum loop is found more suitable for taking the small amount of exudate from the urethra or cervix.

For culture examination the exudate should be rolled out on part of the agar surface as soon as it is obtained. Care must be exercised in rolling the swab over the surface so as not to penetrate the relatively soft agar. If agar plates cannot be supplied to the clinician, the swabs may be introduced into sterile test tubes containing a little broth.

The specimen should be sent at once to the laboratory. If the clinician inoculated the plates, they are further streaked in the laboratory or, if swabs are sent in carrying tubes, plates are immediately inoculated and streaked. The longer the time interval between collection and inoculation of the specimen on the culture medium, the greater the decrease in the number of positive cultures. If cultures are collected sporadically over a period of several hours, they should be placed in a closed candle extinction jar.

For the primary cultivation of specimens from the urethra, cervix, vagina, or rectum, the Thayer-Martin medium selective for gonococci is recommended. Antibiotic supplements of polymyxin B-ristocetin or of vancomycin-colistin-nystatin for use in the selective medium may be obtained from commercial sources.

Subcultures of oxidase-positive colonies of gram-negative diplococci should be made on chocolate agar slants in screw-capped tubes and incubated for 18 to 24 hours in a candle extinction jar. After this time, if gram-negative diplococci in pure culture are present, the cap should be tightened and the tube mailed to the State health laboratory for identification or transmittal to the Venereal Disease Research Laboratory.

7. *Hemolytic Streptococcus Infections.* Specimens usually submitted are material from the anterior nasal or nasopharyngeal areas, or from the throat, or pus, sputum, spinal fluid, discharges, exudates, urine, blood, and milk. The technic of swabbing an area to be cultured is as important in the isolation of streptococci as is the cultivation of the specimen taken. In streptococcal infections of the upper respiratory tract, the organisms may be cultured from the nose or throat specimen. When the specimen is from the

throat, care should be taken to sample an area of inflammation or exudate. If only one specimen can be taken, it should be from the throat. In *carrier* surveys, nasopharyngeal specimens are preferable. Unless a suitable transport method is used, swabs should be planted within not more than four hours after the specimen is taken. All other specimens should be refrigerated from the time taken until they are cultured.

a. **Specimens for isolation of the agent**

Initial isolation of streptococci should, if possible, be carried out in a competent laboratory close to the source of the specimen.

(1) Anterior Nasal, Nasopharyngeal, and Throat Specimens:

Material taken from the upper respiratory passages is the most common type of specimen cultured for hemolytic streptococci. Individual sterile swabs are used and anterior nasal specimens are obtained by introducing the swab into the nares for about an inch. To obtain a nasopharyngeal specimen, the tip of the nose should be elevated, and the swab, moistened with broth or saline, should be introduced along the floor of the nasal cavity, under the middle turbinate, to the pharyngeal wall. Be sure to touch any exudate present. With the tongue depressed, pass a dry swab over the tonsils and pharynx, being sure not to touch the tongue. If inoculation of an isolation medium must be delayed beyond four hours, use one of the following transport methods:

(a) The swab may be inserted into a sterile screw-cap tube containing indicator silica gel.

(b) A blood agar slant in a screw-cap tube may be streaked with the swab which is then left in place during transport.

(c) Several filter paper transport kits are available from commercial sources. The swab should be rolled and scrubbed onto the piece of filter paper and discarded. The filter paper should be allowed to air dry for 3 to 4 minutes; it should then be refolded into its carrier paper, and returned to the envelope.

(2) Pus, Sputum, Spinal Fluid, Discharges, Exudates, Urine:

A Gram stain of the specimen at the local or hospital laboratory will indicate roughly the number of organisms present, and if there are only a few, the fluid may be centrifuged and the sediment cultured. Streak the blood agar plates so as to obtain well-isolated colonies and inoculate a tube of enriched infusion broth.

(3) Blood

After decontaminating the skin with 4% iodine followed by wiping with 70% alcohol, use a sterile syringe to draw about 10 ml. of blood and add it to a flask containing 2 ml. of 3% sterile sodium citrate or 1.0 mg. of heparin. After mixing, transfer the specimen aseptically to 100 ml. of blood culture broth. A Loeffler slant or a blood agar plate may also be inoculated. If a plate is used, limit the inoculum to a small area of the medium and take the plate to the laboratory where streaking for isolation can be done properly with a wire loop. *Do not mail Petri dishes.* The addition of antagonists to media for *blood cultures* from patients undergoing chemotherapy and the addition of penicillinase in cultures from patients receiving penicillin are usually unnecessary.

(4) Milk

Refrigerate the specimen for as much of the time as possible before it is cultured. If refrigeration is impossible, mix the specimen with ⅓ volume of glycerol. Culture suitable dilutions in blood agar pour plates in duplicate, mixing the sample and melted medium in the plate so as to get isolated colonies. Incubate one plate aerobically and one under anaerobic conditions.

b. **Streptococcus grouping by fluorescent antibody (FA) technic**

Smears made directly from the patient are unsatisfactory for examination by FA technic. For this examination, specimens may be transported in the same manner as for culture. The swab should be placed in Todd-Hewitt broth for enrichment purposes for two to three hours at 37°C. before the FA smears are made.

c. **Specimens for streptococcus typing**
Very few laboratories are in a position to type streptococci. If isolates are to be sent to the NCDC for typing, they should be submitted only after consultation with the Streptococcus Unit of the Laboratory Program and then, as pure cultures on blood agar slants.

8. *Haemophilus Infections of the Pharynx or Conjunctiva*
Specimens for the isolation of the agent
Blood, sputum, throat, or nasopharyngeal swabs, and purulent exudates are collected in the customary manner, using aseptic technic where feasible. Since use of swabs made of wooden sticks or wire inhibit the growth of these organisms, small swabs made of cotton attached to some inert, synthetic material such as nylon or Teflon, which is not inhibitory, should be used. Teflon tubing has proved to be satisfactory for this purpose. A small, sterile, dry swab may be rotated gently over the conjunctiva or pharyngeal mucosa to obtain some of the exudate if any is present. The swab should be immediately placed in a tube of semisolid agar for transport to the laboratory where it is incubated for about 4 hours. Plates of transparent agar and blood are then streaked for isolation of the organism. Blood should be inoculated into a flask of broth with a large surface area. Sputum and throat specimens are cultured on one of the transparent agars as well as on blood agar. The swab should not be left in the tube of semisolid agar during incubation.

9. *Leptospirosis.* This disease should be considered in all cases of febrile illness of unknown origin and it may suggest aseptic meningitis, or non-paralytic poliomyelitis. Rats, dogs, cattle, swine, and many wild animals are common animal reservoirs. Infection is transmitted to man by urine containing the agent and occurs when leptospires enter the body through the mucous membranes of the mouth, nose, throat, eyes, lungs, or abraded skin.
a. **Specimens for the isolation of the agent**
Blood, urine, and cerebrospinal fluid may be collected for culture and/or animal inoculation with a view to recovery of the organism. Blood taken during the febrile stage of illness is the most reliable for culture, but spinal fluid collected within the first 10 days of illness also may be cultured. Inoculate the freshly-drawn specimen directly

into several tubes of a suitable semisolid medium such as Fletcher's. Multiple tubes of media should be used and small inocula, 1 to 3 drops of blood to 5 ml. of medium, planted. As Fletcher's medium remains stable for 3 to 4 months, the laboratory may supply it to the physician. It can be inoculated at the bedside, and then shipped by mail. Voided urine if diluted, may be cultured directly into semisolid medium. Five 10-fold dilutions in buffered saline or a suitable broth are first prepared. Since quantitative dilutions are not necessary, a single 2.0 ml. syringe with a 20-gauge needle may be used to prepare these dilutions in the syringe. Draw up to 0.1 ml. of urine and 0.9 ml. of diluent for the first dilution; expel all but 0.1 ml. of this dilution, planting one drop into 5 ml. of medium. For the second dilution, again draw 0.9 ml. of diluent into the syringe. Plant one drop of the mixture into 5 ml. of medium again expel all but 0.1 ml. Repeat with the third, fourth, and fifth dilution. Otherwise, inoculate into such test animals as weanling hamsters or guinea pigs.
Any pure cultures isolated should be shipped in semisolid media to a reference laboratory for confirmation by serologic tests.
b. **Specimens for serologic tests**
Microscopic or macroscopic agglutination tests are the most common serologic procedures used for the diagnosis of leptospirosis. To detect a rise in titer, a specimen of blood should be taken at two different times: one during the first week or acute phase of illness, the other ten days or two weeks later. Maximum titers are usually reached by the third or fourth week. While serodiagnostic tests are of value in confirming past or current leptospiral infection, paradoxical reactions may occur, and determination of the infecting serotype can be made only by isolation and serologic identification of the leptospires.

10. *Meningitis.* Any organism capable of invading human tissues can cause infection of the meninges. As used here, the term "meningitis" refers to an inflammation of the meninges, brain, or spinal cord associated with increased cell counts in the spinal fluid. Characteristic of spinal fluid in bacterial meningitis is a predominance of polymorphonuclear cells and a decrease in dextrose content.

Representatives of the following species or groups of bacteria are most often involved in meningitis: 1) *Neisseria meningitidis,* 2) *Haemophilus influenzae,* 3) *Diplococcus pneumoniae,* 4) *Streptococcus pyogenes,* 5) *Staphylococcus aureus,* 6) *Proteus* species, 7) *Pseudomonas* species, 8) *Escherichia coli,* 9) *Mycobacterium tuberculosis,* 10) *Listeria monocytogenes,* 11) *Mimeae,* and 12) *Flavobacterium meningosepticum.* Meningitis may also be caused by viruses, fungi, spirochetes, or protozoa.

Specimens for the isolation of the infectious agent

Spinal fluid, blood, nasopharyngeal swabs, petechial scrapings, and less frequently ventricular, cisternal, or subdural fluid, are usually submitted for laboratory examination. The time in the course of the infection at which the specimen is taken, the temperature at which it is held, and the amount of the inoculum used for cultures are all important. Spinal fluid should be taken as soon as meningeal symptoms appear; blood for cultures, as soon as infection is suspected; petechial scrapings for culture, in either doubtful or postmortem cases. Nasopharyngeal swabs are cultured for carrier detection. All specimens for isolation of the meningococcus should be transmitted to the laboratory with a minimum of delay. If nasopharyngeal swabs are not planted on plating media immediately, they may be transported in a tube containing 0.5 ml. heart infusion broth. Blood for culture may be drawn directly into a vacuum bottle containing appropriate medium and taken to the laboratory for incubation.

11. *Plague.* From time to time epidemics of plague have swept over large areas of the world, temporarily paralyzing all forms of human activity. Plague is primarily a disease of rats and wild rodents. It is transmitted from animal to animal by the bites of infected fleas. Man serves only as an accidental host. The pneumonic type of the disease can, however, be spread from man to man by droplet infection without the intervention of an insect vector.

a. **Specimens to be collected for isolation of the agent**
Bubo fluid, portions of bubo, spleen, bone marrow, sputum, blood, or ectoparasites may be submitted for cultures in cystine broth or on blood agar slants or plates. Do not ship plates. Original specimens should be shipped in double containers with screw tops. The disease may be identified in decayed or mummified carcasses by precipitin tests or the fluorescent antibody technic.

b. **Diagnosis of plague by FA technic**
This procedure appears to be reliable and rapid yielding results in one hour, in the examination of bubo exudate, blood from human cases, tissue impression smears, or cultured organisms. However, if clinical material is injected into laboratory animals, about two days must be allowed for the development of organisms within the animal before the FA test may be made or isolation by culture begun.
Plague bacteriophage may be used to differentiate between plague and pseudotuberculosis organisms.

12. *Syphilis.*

a. **Guide for submitting blood specimens for the *Treponema Pallidum* Immobilization (TPI) test**

(1) Criteria for requesting the TPI test
This test should be requested only on specimens from patients who are diagnostic problem cases: (a) with reactive nontreponemal tests and no history or clinical evidence of syphilis; or (b) with nonreactive nontreponemal tests and suggestive evidence of syphilitic infection.

(IF THE PATIENT HAS RECEIVED ANY INJECTED ANTIBIOTICS WITHIN ONE MONTH OR ORAL ANTIBIOTICS WITHIN ONE WEEK OF THE DRAWING OF THE BLOOD SPECIMEN, AN INVALID OR INCONCLUSIVE FINDING IN THE TPI TEST MAY RESULT.)

(2) Collection of the blood specimen
(a) Collect at least 5 ml. of blood with a sterile syringe and needle.
(b) Transfer the blood to a sterile test tube and stopper with a *paraffin-coated cork.* Uncoated corks or rubber stoppers are unsatisfactory.
NOTES:
Unless a vacuum tube with a nontoxic rubber stopper is used, the rubber stopper should be replaced immediately after collection of the specimen. Vacutainer tubes with nontoxic rubber stoppers are available from Becton-Dickinson (Catalog #4719, description #3200 NT.)

13

BACTERIAL CONTAMINATION REND-
ERS SPECIMENS UNSATISFACTORY FOR
TESTING. SERA SHOULD *NOT* CONTAIN
PRESERVATIVE OR ANTICOAGULANT.

    (c) Secure the stopper in the tube with adhesive tape.

    (d) Place the blood specimen in a mailing container with the completed clinical history form and mail it immediately to the State public health laboratory.

    (e) A clinical history form on the patient should be submitted to the State laboratory with the specimen. This should include information on evidence or history of syphilis, other treponematoses or venereal diseases in the patient or family, previous therapy, a record of previously performed serologic tests for syphilis on serum or spinal fluid specimens, and evidence of diseases or conditions other than syphilis.

(3) Preparation of the sterile serum sample

    (a) The State public health laboratory will *aseptically* separate and transfer the serum to a sterile tube stoppered with a paraffin-coated cork.

    (b) The sterile serum sample will then be forwarded immediately to:

    The National Communicable
    Disease Center
    Venereal Disease Research
    Laboratory
    Atlanta, Georgia 30333

(4) Reporting of results
Test results will be reported to the State public health laboratory for forwarding to the submitting physician.

(5) Public Health Service facilities
These facilities have special instructions and forms for submitting specimens directly to the Venereal Disease Research Laboratory.

b. **Darkfield examinations for** *Treponema pallidum*
Darkfield examination is an examination of exudate from suspected syphilitic lesions with a compound microscope equipped with a darkfield condenser. The darkfield examination should be performed on any rash or lesion suspected of being syphilitic, and, ideally, it should be performed immediately after the specimen is collected. If this examination cannot be performed locally, contact the State department of public health laboratory and/or the venereal disease control section for information on the availability of this service.

c. **Fluorescent antibody darkfield (FADF) examination for** *T. pallidum*

    (1) Clean the suspected syphilitic lesion with a gauze sponge wet with tap water or saline. Dry the area and abrade it with a dry sponge. It may be necessary to squeeze the base of the lesion to promote the appearance of serum. For dry lesions, apply a large drop of saline and emulsify the surface material with a toothpick or bacteriological loop.

    (2) Collect the specimen in a dry capillary tube (optimum dimensions — 75 x 1.5 mm.).

    (3) With a 70%-alcohlol sponge, wipe off the top of the capillary tube which contacted the lesion and seal both ends of the tube with clay, paraffin, Critocaps, or a small flame.

    (4) For mailing specimens to a central laboratory, place the capillary tube in a cardboard slide-mailing container, seal with cellophane or masking tape, **OR**, place the capillary tube in a test tube, stopper, place in a mailing container, and pack to protect against breakage.
FREEZING SPECIMENS BEFORE MAILING TO A CENTRAL LABORATORY IS NOT NECESSARY.

    (5) When specimens are received in the laboratory for examination, the capillary tube (in the mailing container), plus the identification slip, is placed in a freezer ($-20°$C. to $-40°$C.) until the specimen is completely *frozen*. If the specimen is to be examined immediately, the capillary is removed from the mailing container for more rapid freezing. After freezing, thaw the specimen at room temperature and examine it according to the FADF procedure.

d. **Blood specimens for the nontreponemal**

tests, the one-fifth volume Kolmer with Reiter protein antigen (KRP) and Fluorescent Treponemal Antibody (FTA) tests.

(1) Collection tubes should be clean, dry, and sterile to prevent contamination and hemolysis of the specimen. Vacuum tubes or tubes with paraffin-coated corks may be used.

(2) At least 5 to 8 ml. of blood should be drawn, placed in the tube aseptically, and allowed to clot at room temperature. Store the specimen in the refrigerator until the specimen is sent to the laboratory. Specimens should not be placed in the mail over long weekends or holidays when delivery may be delayed.

NOTE: Hemolysis may be caused by wet or dirty syringes, needles, or tubes; chemicals; freezing; or extreme heat.

(3) If serum is submitted to the laboratory, submit information as to whether or not it has been heated (giving time and temperature) and if preservatives have been added.

e. **Spinal fluid specimens for nontreponemal tests and total protein determinations**

(1) Collection tubes should be clean and sterile. (Merthiolated tubes may be prepared for the collection of the specimens: prepare a 1% aqueous solution of Merthiolate; place 0.1 ml. in a clean, sterile tube; and dry in a desiccator over $CaCl_2$. This compound curtails bacterial growth without interfering with the nontreponemal tests for syphilis and does not affect the results obtained with the turbidimetric methods for determining total proteins in spinal fluids.) Stopper with paraffin-coated corks.

(2) Collect 2 to 8 ml. of spinal fluid aseptically.

(3) If the specimen is centrifuged before sending it to the laboratory, note the original condition or appearance of the specimen on the request slip.

NOTE: Specimens grossly contaminated with blood or bacteria are unsatisfactory for testing.

13. *Tuberculosis.* Since *Mycobacterium tuberculosis* may invade any organ of the body, such varied specimens as sputum, gastric washings, pus, urine, or spinal fluid may be sent to the laboratory for examination. Sputum is the specimen most frequently submitted. Patients must be taught the difference between saliva and sputum and told that an early morning specimen is generally most productive. They should also be cautioned against contaminating the exterior of the container, as such contamination creates a hazard for all who handle the specimen.

Pooling of sputum for several days is not desirable because it may be toxic to tubercle bacilli present and the degree of contamination may be increased. Specimens must be sent through the mails in sterile screw-capped containers having resilient rubber liners in the caps. Packaging must be in double mailing containers. The need for examination of repeated specimens should be stressed, and the results of a single negative specimen should never be accepted as conclusive evidence of the absence of disease. *Specimens submitted in unsterile containers, pill boxes, fruit jars, ointment jars, or in paper envelopes or on pieces of gauze are utterly useless and will not be examined by the laboratory.* Pathogenic fungi capable of causing pulmonary mycosis are sometimes encountered during culture work for tuberculosis. While certain saprophytic fungi also survive the processing of specimens, none of the fungi isolated should be casually discarded as harmless contaminants; instead, they should be referred to a competent mycology laboratory for identification.

Specimens to be collected for isolation of the agent

a. Sputum, the thick yellowish-green exudate from the lungs, and not saliva, should be collected in whatever type of sterile container is furnished by the laboratory, and in no other. A pinch (about 50 mg.) of sodium carbonate added to the container will help to suppress the multiplication of contaminants. Be sure the sputum is deposited within the container without soiling the exterior. Make sure the closure is leakproof and forward the specimen to the laboratory in the double mailing container.

b. Gastric washings, collected in the morning on a fasting stomach, should be transported to the laboratory as promptly as feasible. The more prompt the examination the greater the chance of recovering the tubercle bacillus. If the specimen is to be sent through the mails, some attempt should be made to

15

neutralize the acidity with buffering tablets or sodium carbonate.

c. Urine may be contaminated with acid-fast saprophytes present on the external genitals, so a catheterized specimen is preferable. If catheterization is impractical, a midstream sample, collected in a sterile container, after careful washing of the external genitals, may be acceptable.

d. Other materials such as samples of spinal, pleural, or synovial fluid, and pus, may be collected for diagnostic examination. Aseptic collection in sterile tubes and the addition, where indicated, of an anticoagulant such as ammonium oxalate, are essential to maintain the specimen in a fluid state.

14. *Tularemia.* The laboratory diagnosis of tularemia may be accomplished by either a) the agglutination test, or b) cultural examination. The extreme infectivity of *Pasteurella tularensis,* however, makes routine culture inadvisable, and agglutination tests are the method of choice in the diagnostic laboratory.

a. **Specimens for isolation of the agent**

Culture of *P. tularensis* from any one of the natural hosts such as ground squirrels, wild rabbits, wild mice, quail, or other animals should not be attempted *except in a reference laboratory.* If such an animal is shipped, the body should be wrapped in cloth soaked in cresol, placed in a container with dry ice, and forwarded to the laboratory immediately. Identification of the organism in smears made from the blood or tissues of the host is not possible; but in human cases, cultures may be made from any of the organs or from the sputum, blood, or exudates, and the organism may be identified by specific agglutinating antisera. Specific FA conjugate may also be used.

b. **Specimens for serologic tests**

Blood should be drawn aseptically, allowed to clot, and the serum removed for agglutination tests. Care should be taken to assure that the blood is not hemolyzed due to exposure to excessive heat or cold in transit to the laboratory. To demonstrate a rise in antibody titer, preferably at least two blood specimens should be obtained—the first during the acute stage of the disease, and the second about three weeks later. Precipitin tests are also possible; but these, together with FA tests, probably will be available only in a reference laboratory.

MYCOTIC DISEASES

Fungus diseases are conveniently classified as cutaneous, subcutaneous, and systemic infections. The etiologic agent is identified by study of its morphology as seen in clinical material and by its morphological and physiological characteristics as observed in pure culture. Serological methods, for example, agar gel precipitin tests, agglutination tests, and FA techniques, are also of value in the identification of certain pathogenic fungi.

Specimens taken for laboratory examination vary with the disease and the tissues involved. Specimens include sputum, gastric washings, spinal fluid, blood smears, citrated blood, blood serum, bone marrow, pus, pleural fluid, urine, biopsy or surgical specimens, scrapings from edges of lesions, skin scrapings, hair, and nail clippings. If competent local laboratory assistance is available, it should be used for the proper collection of clinical materials. Since many clinical materials such as sputum, lesion exudates, and tissues are readily overgrown by contaminating bacteria and saprophytic fungi, it is usually wise to make cultures on selective and nonselective media and to ship these, rather than the clinical materials, to the reference laboratory. The use of penicillin and streptomycin or chloramphenicol as antibacterial inhibitors in the specimen may be helpful if cultures cannot be made before shipment.

It must be borne in mind, however, that certain of the pathogenic fungi, particularly *Nocardia asteroides* and *Actinomyces israelii,* are sensitive to antibacterial antibiotics. Furthermore, others, particularly *Histoplasma capsulatum,* die out rather quickly in clinical material. Refrigeration of the specimen in transit will help to overcome this difficulty. In general, refrigeration of specimens in transit serves not only to maintain viability of fungus pathogens but also to decrease growth of contaminants.

1. *Cutaneous fungus infections.* Careful choice of specimens for laboratory study is important. The Wood's lamp is useful in the collection of specimens in tinea capitis infections since hairs infected by most members of the genus *Microsporum* frequently exhibit fluorescence under a Wood's lamp. However, in tinea capitis due to *Trichophyton* species, infected hairs usually do not fluoresce.

a. **Specimen collection for laboratory examination**

Fluorescent hairs, or nonfluorescent hairs which are broken off and appear diseased, should be plucked with a sterile forcep, or, if diseased hair stubs are not

apparent, the edges of a scalp lesion should be scraped with a sterile scalpel. Skin lesions should first be cleansed with 70% alcohol to reduce bacterial and saprophytic fungi. Scrapings should be made from the outer edges of skin lesions. In infections of the nails, the friable material beneath the edge of the nails should be scraped out, or portions of abnormal appearing nail should be scraped or clipped off, and saved for examination and culture.

Enclose hair specimens, skin scrapings, or nail clippings or scrapings in clean paper envelopes and label them with the patient's name or specimen number. Enclose these envelopes in larger heavy paper envelopes for mailing to the laboratory. *Do not put specimens in cotton plugged tubes,* as the specimen may become trapped among the cotton fibers and lost. *Do not put specimens into closed containers such as rubber stoppered tubes* as this keeps the specimen moist and allows overgrowth of bacteria and saprophytic fungi.

2. *Subcutaneous fungus infections.* Procedures for isolating the etiologic agents of chromoblastomycosis, mycetomas, and sporotrichosis are obvious. Granules, pus, or serosanguinous fluid from lesions or draining sinuses should be inoculated on several tubes of Sabouraud dextrose agar with and without chloramphenicol and cycloheximide.

3. *Systemic fungus infections.* Systemic mycoses are not contagious and most are chronic and evolve slowly. Since time is usually not of vital importance, it may be profitable to consult with a mycology laboratory to determine what type of specimen to collect in a given case, and how it should be handled. Examining unstained preparations of clinical material as well as stained smears is of practical value in determining the appropriate types of media to inoculate and the correct incubation temperatures. It is usually wise to inoculate both simple media, such as Sabouraud dextrose agar, and enriched media, such as brain heart infusion agar. Each medium should be made with and without antibiotics.

Infections with *Histoplasma capsulatum* and *Coccidioides immitis* present special problems to the laboratory worker. In the case of histoplasmosis it is often difficult to isolate the causative fungus because the organisms are often few in number and tend to die out in the clinical materials. In the case of coccidioidomycosis the fungus agent is relatively easy to isolate. It is highly infectious, however, and many laboratory workers hesitate to attempt its isolation. Suggestions on methods for the isolation and handling of *H. capsulatum* and *C. immitis* are given below.

a. Actinomycosis

If isolates of suspected anaerobic to microaerophilic *Actinomyces* species are to be sent to a reference laboratory for identification, the following methods are recommended.

(1) Liquid media

(a) Inoculate culture into freshly boiled TST enriched* thioglycollate broth. Incubate at 37° C. for 3 to 4 days. If growth is apparent at that time, seal by pouring over the surface of the medium about 3 ml. of a sterile melted mixture of paraffin and vaseline (50% paraffin & 50% vaseline).** Seal the top of the tube with a sterile rubber stopper, or use a screw-cap tube sealed with masking tape.

(b) If Actinomyces Maintenance Broth*** is used, inoculate the tube of broth and incubate under pyrogallol-carbonate seal for 3 to 4 days. If growth is apparent, transfer the liquid with the culture to a clean sterile tube, and seal with vaseline-paraffin mixture as descibed above.

THE IMPORTANT POINTS TO REMEMBER ARE:

(1) Actinomyces cultures should not be more than 3 to 4 days old before shipment. Send them by airmail if possible.

(2) Liquid or semisolid cultures, should be sealed with vaseline-paraffin mixture before shipment.

* Thioglycollate with TST
  Thioglycollate broth
    (with dextrose and indicator)   29.5 gms.
  Trypticase Soy broth   1.5 gm.
  Tryptose broth   1.25 gm.
  Distilled water   1000.00 ml.

** A stock vaseline-paraffin mixture may be prepared by melting together equal amounts of each. After mixing well, it may be distributed in 3 ml. amounts in plugged tubes for sterilization. These may be stored for use as needed.

*** This medium is recommended for fastidious strains, which do not grow well in thioglycollate broth. It is available in desiccated form from BBL, Division of Bio Quest, P. O. Box 175, Cockeysville, Md., 21030.

b. Coccidioidomycosis

Direct microscopic examination of wet, unstained clinical material should be made, but, regardless of the direct findings, cultures and/or animal inoculations are indicated. *Safety precautions: Petri plate cultures should never be made* because they may lead to laboratory infection. Cultures should be in large test tubes which give broad agar surfaces for inoculation. Specimens held for any length of time, or shipped to the laboratory, should be in tubes containing an antibiotic to retard growth of contaminating organisms. *If the laboratory worker does not have the proper experience or equipment for handling the highly infectious cultures of C. immitis,* the animal inoculation or "indirect method" for demonstration of the fungus should be used (see below).

(1) **Specimen collection for laboratory examination**

(a) Merthiolated serum from serial blood specimens should be examined by the complement fixation test. Add merthiolate to retard growth of contaminants.

(b) Sputum, pus, pleural fluid, and gastric washings treated with the antibacterial antibiotics, chloramphenicol or a combination of penicillin and streptomycin, may be inoculated directly into mice or cultured on a selective medium such as that containing cycloheximide and chloramphenicol. Preliminary identification of cultures by microscopic examination must be confirmed by animal inoculation. *Unless safety hoods are available, however, it is advisable to use the animal inoculation or "indirect method" for the demonstration of C. immitis. By this method, the handling of the highly infectious mycelial phase of this fungus is avoided.*

(2) **Animal inoculation of cultures suspected of being *C. immitis* and the "indirect method" for demonstration of *C. immitis* from the clinical materials.** Mice may be inoculated intraperitoneally and guinea pigs intratesticularly with culture suspensions, or directly with clinical materials which have been treated with antibacterial antibiotics. The male guinea pig is the animal of choice in determining whether a mycelial culture is or is not *C. immitis,* or whether the clinical material contains elements of this fungus. Intratesticular injection of these animals with *C. immitis* gives rise to an orchitis and examination of fluid withdrawn from the testes will reveal the tissue form of *C. immitis.* If guinea pigs are not available, inject mice intraperitoneally and examine lesions or lymphatic exudates for the fungus.

c. Histoplasmosis

If possible, culture media should be inoculated immediately following collection of clinical material. If the specimens are to be shipped or held for any length of time, they should be refrigerated because these organisms die out rapidly when exposed to higher temperatures. An antibiotic such as chloramphenicol should be added to specimens that are to be shipped.* Recovery of *H. capsulatum* by direct culture from clinical materials is not always difficult, but the chances of recovery are greatly increased if mice are inoculated and sacrificed for culture at the end of two weeks.

(1) **Specimen collection for laboratory examination**

(a) Blood smear examination is unprofitable except in acute, disseminated cases.

(b) Citrated blood is of value for culture and animal inoculation in acute, disseminated cases.

(c) Merthiolated** serum from serial blood specimens, taken at 3- to 4-week intervals, should be tested by complement fixation and agar gel precipitin tests.

(d) Sputum specimens*** should be collected in sterile bottles to which chloramphenicol has been added. A minimum of six specimens should be collected from each patient before histoplasmosis is ruled out.

* See appendix II, page xx
** See appendix II, page xx
*** Sputum brought up from bronchial areas by having the patient use correct postural drainage procedures.

18

(e) When sputum cannot be obtained, gastric washings may be submitted. They should be collected in a sterile bottle containing chloramphenicol.

(f) Spinal fluid is taken only if there is cerebral meningeal involvement. It is collected in a tube or bottle containing antibiotic.

(2) **Animal inoculation**

Intraperitoneal inoculation of mice with clinical materials greatly increases the probability of demonstrating the presence of *H. capsulatum*. Sacrifice the animals after 2 weeks and culture the liver and spleen on cycloheximide agar at room temperature and on blood agar without antibiotics at 37° C.

d. Fluorescent Antibody (FA) Staining in Mycology

In certain reference laboratories, FA technics are regularly used in conjunction with conventional methods of identification. Currently these procedures have been most successfully applied in medical mycology in the identification and detection of *Sporotrichum schenckii*, *Blastomyces dermatitidis*, *Cryptococcus neoformans*, *H. capsulatum*, and *C. immitis*.

Specimens for examination by the fluorescent antibody technique are obtained from such varied sources as blood, spinal fluid, sputum, lesion exudates, sternal marrow, scrapings from edges of ulcers and abscesses, and aspirated fluid from abscesses and tissue. If the laboratory is close by, such specimens can be submitted directly to the laboratory. If these materials have to be shipped to a distant laboratory, smears should be prepared for shipment. Sputum should be enzymatically digested (by parasentin or trypsin) and smears made from the centrifuged sediment. Such smears are made directly within etched circular (1 cm. di.) areas of glass slides and are then allowed to air dry. These preparations are fixed by heat. Other materials such as exudates or tissues do not require digestion. These smears are made directly within the etched areas of glass slides, are allowed to air dry and are then fixed by heat. Spinal fluid or gastric washings should be *centrifuged* and smears made from the sediment.

PARASITIC DISEASES

The diseases caused by animal parasites may be conveniently considered as blood, intestinal, or tissue infections; and laboratory diagnosis may be made by direct examination of the specimen or indirectly by serologic tests. The specimens submitted for laboratory examination may be smears of bone marrow, liver, spleen, or whole blood, aspirates from lesions, vaginal or urethral secretions, bile, sputum, duodenal drainage, anal swabs, feces, or portions of the parasite itself.

1. *Blood Parasites.* Proper collection and handling of specimens to be examined for blood parasites are important since inadequate or poor samples may lead to erroneous conclusions. Not all the organisms usually grouped as blood parasites are diagnosed from blood. In certain instances, spinal or peritoneal fluid, aspirates and biopsies of organs and tissues are also used. The specimen to be obtained depends on the location of the parasite or its diagnostic stage in the body. Table 1 shows the location of parasites or their diagnostic stage within the body.

TABLE 1

Location of Parasites or their Diagnostic Stage within the Body

	PERIPHERAL BLOOD			OTHER			
	Within RBC	In Plasma	Within Leuco-cytes	Skin	Bone Marrow	Lymph Nodes	Spinal Fluid
Plasmodia spp. (4)	+				+		
Trypanosoma gambiense and T. rhodesiense		+				+	+
Trypanosoma cruzi		+				+	
Leishmania donovani			+		+	+	
Leishmania tropica and L. brasiliensis				+			
Filaria-microfilariae (5 spp.)		+			+*	+**	
Onchocerca volvulus				+			
Toxoplasma gondii			+			+	+

*Loa loa microfilariae may be found in calabar swellings
**Wuchereria bancrofti and Brugia malayi microfilariae

a. Blood specimens
Two types of specimens are collected for recognition of those parasites whose diagnostic stages are found in peripheral blood, (1) dried blood films for staining and (2)

whole blood samples. This applies in malaria, trypanosomiasis, and filariasis (with the exception of onchocerciasis).

(1) Blood films

Citrated, oxalated, or clotted blood should not be used to make blood films except in emergencies. In *thin films* the blood is spread over a relatively large area in a thin layer; in *thick films,* it is concentrated in a small area. For routine diagnosis, the thick film is preferable since it permits the examination of a large amount of blood. However, parasite morphology is more distinct and typical in a thin film. For this reason, a thin and thick film combination on the same slide is recommended.

Thin films are prepared by pricking the ear or finger after the area has been cleansed with gauze, *not cotton,* soaked in 70% alcohol, and allowed to dry completely. A single drop of blood is deposited near one end of a slide and spread as though making a preparation for a differential count. *Thick films* are made by either touching the under surface of the slide to a fresh large drop of blood on the finger, without touching the skin, and rotating the slide to form a film about the size of a dime. Alternatively, several drops of blood may be deposited close together near one end of the slide and puddled with the corner of a slide, applicator stick, or toothpick. The films are allowed to air dry in a horizontal position protected from dust and insects. Thin films dry rapidly but thick films require 8 to 12 hours. Properly and improperly prepared films are shown in Figure 1.

(2) Whole blood

If concentration methods are to be used, blood is collected by venipuncture, and sodium citrate or heparin is added to prevent clotting. Aseptic technic is essential in the collection of the sample. Do not use preservatives since they may kill the organism and make cultivation or animal inoculation impossible.

(3) Time of collection

Time of specimen collection in malaria is important but less so than in filaria infections. Parasites are most numerous in malaria about midway between chills, but their morphology is somewhat less characteristic. One specimen taken at this time and a second 5 to 6 hours later is ideal.

Because of the nocturnal periodicity in certain filaria infections, notably by *Wuchereria bancrofti* and *Brugia malayi,* the specimen should be taken between 10 p.m. and 2 a.m. Infections with *W. bancrofti* or *Wuchereria pacifica* in the southwest Pacific area exhibit no such periodicity. In *Loa loa* there is a diurnal periodicity, and specimens should be collected between 10 a.m. and 2 p.m. Blood collection should also be correlated with the stage of the infection. For example, *Trypanosoma cruzi* is found in the blood only during the acute, febrile stage of Chagas' disease. The same is true for infections with *Trypanosoma gambiense* and *Trypanosoma rhodesiense.*

b. Body fluid, aspirates, and biopsies

Certain blood parasites are diagnosed by the examination of body fluids and tissues rather than by direct examination of blood. Thus, biopsies of liver, spleen, bone marrow, and lymph glands must be studied to demonstrate the parasite. This is true in leishmaniasis, filariasis (onchocerciasis), and sometimes in malaria and trypanosomiasis. In some cases of onchocerciasis, tissue biopsies may reveal microfilariae when examination of skin from the nodule is negative. Localization of the nodules on the upper or lower part of the body influences distribution of the microfilariae and dictates selection of the biopsy site which may be the shoulder, calf, thigh, or another part of the leg.

Aspirates of lymph glands or ulcerative skin areas are collected for diagnosis of leishmaniasis, trypanosomiasis, or filariasis. Cerebrospinal, hydrocele, pericardial, pleural, and peritoneal fluids may be used for the diagnosis of trypanosomiasis, filariasis, or toxoplasmosis.

Handling of tissues or fluids depends on the examination to be made. If cultures or animals are to be inoculated, asepsis should be practiced, and the media or animals should be inoculated as soon as possible after the specimen is collected. If inoculation is delayed, the specimen should be stored in the refrigerator.

Figure 1
## PROPERLY MADE SLIDES



Thick and thin film

Thick film only

Errors to be avoided

Grease on slide
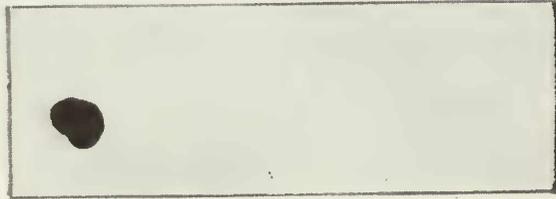
Too small
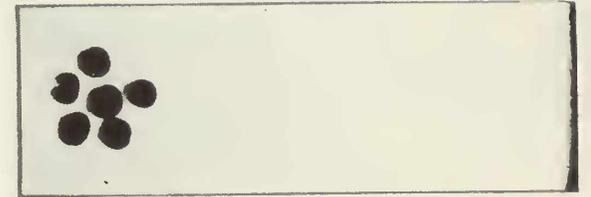
Drops not connected

Too thick

Improperly dried

Wrapped while wet

Too thin

Improperly placed

Damaged by flies

21

cox

Tissue for sectioning should be placed in a fixative as soon as it is removed from the body.

c. Shipment of specimens

Dry blood or tissue smears may be placed in slide boxes with tissue between and over the slides to prevent breakage. Slides may be wrapped in bundles, slide over slide, in toilet tissue with one or two layers of tissue between adjacent slides. The slides may be packed in a mailing container or box with enough protective material to prevent breakage, but these containers should be packed in a box with additional packing material.

d. Storage of specimens

Prompt examination of blood specimens is desirable because blood loses its affinity for stain after 3 to 4 days. Unstained slides may be refrigerated a week, but, if staining is to be delayed, thin films should be fixed with methyl alcohol and thick films dehemoglobinized in buffered water before storage. Subsequent staining requires special technic. Prompt refrigeration of whole blood is necessary if it is not to be examined immediately. Microfilariae remain alive in blood a week or more under refrigeration. Tissue or fluid smears, like thin blood films, may be stored in the refriegrator, after fixation with methyl alcohol, before being stained.

Stained blood films, tissue impression smears, or fluid smears may be preserved by covering the film with a coverslip or a coating of clear Diaphane or other neutral mounting medium.

e. Immunodiagnostic tests in parasitic diseases

The immunodiagnostic tests employed in parasite diagnosis are, in general, modifications of procedures in common use, that is, complement fixation, precipitin, hemagglutination, flocculation, or fluorescent antibody technics.

Various types of immunodiagnostic tests and the present status of their applicability in a variety of parasitic diseases are shown in Table 2. Specimens collected for serologic diagnosis of parasitic infections are taken as for other types of serologic tests. The serum should never be inactivated. If examination is to be delayed, 0.01 ml. of 1% aqueous solution of borated merthiolate per ml. of serum may be added (Appendix II). If the serum is to be used also for virus serology, it *must not* be merthiolated.

2. *Intestinal Parasites.* Diseases caused by intestinal parasites are diagnosed mainly by the examination of fecal specimens, but urine or sputum may be used for diseases caused by certain species. Properly collected and preserved specimens are of utmost importance since old, or poorly preserved materials are of little value in establishing a diagnosis and may lead to erroneous conclusions.

a. **Fecal specimens**

The stool should be collected in a clean container or on clean paper and transferred to a suitable container such as a half-pint waxed carton with an over-lapping lid. Urine must be excluded since it will destroy trophozoites if they are present. *Feces deposited on* soil are not satisfactory due to the possible presence of free-living larvae and other contaminants which may confuse the diagnosis. The specimen should be taken to the laboratory at once; or if the examination will be delayed, a small portion, in addition to the carton specimen, should be placed in PVA fixative (polyvinyl alcohol) (Appendix II) to preserve trophozoites. *Administration of barium, magnesia, or oil prior to collection will render a specimen unsatisfactory for examination.*

b. **Mailed specimens**

Specimens sent through the mail must be in containers which meet postal regulations for infectious materials.

One-ounce, screw-cap vials placed in a metal case with a screw cap and enclosed in a cardboard container with a metal screw cap are satisfactory. The vials should be sealed with adhesive tape around the top to prevent leakage. They should then be packed in suitable absorbent materials to prevent breakage from shock transmission and to absorb entirely any leakage that would result if breakage should occur. Mailed specimens require use of a preservative, and a two-vial method of collection and shipping is advocated. One vial contains 5% or 10% formalin, the other PVA fixative. Thus the laboratory has a formalinized specimen that can be examined for cysts and helminth eggs and a PVA-fixed specimen that can be examined for trophozoites, and to a lesser degree, for cysts. The method of handling a stool specimen for parasitological examination is shown in Figure 2.

Table 2
IMMUNODIAGNOSTIC TESTS FOR PARASITIC INFECTIONS

PARASITIC DISEASES \ TESTS	Intradermal	Complement Fixation	Precipitin	Agglutination	Flocculation	Hemagglutination	Latex Agglutination	Fluorescent Antibody	Methylene Blue Dye Test
Ascariasis	◎	◎	◉			◉			
Trichinosis	●	●	●		●	◉	◉		
Toxocariasis	◎				◎	◎			
Cysticercosis		●	●						
Echinococcosis	●	●	◉		●	●			
Schistosomiasis	◉	●	●	◎	◉	◉			
Clonorchiasis	◉	◉				◎			
Paragonimiasis	◉	◉							
Filariasis	◉	◉		.					
Chagas' disease	◎	●	◎	◎				◎	◎
Leishmaniasis	●	◉							
Toxoplasmosis	●	●		◎		◉		◉	●
Amebiasis		◉	◎						

◎ Under experimental investigation
◉ Used for diagnosis but requires further evaluation for routine use
● Generally accepted useful routine diagnostic test

23

Figure 2

PARASITOLOGICAL EXAMINATION OF STOOL SPECIMENS
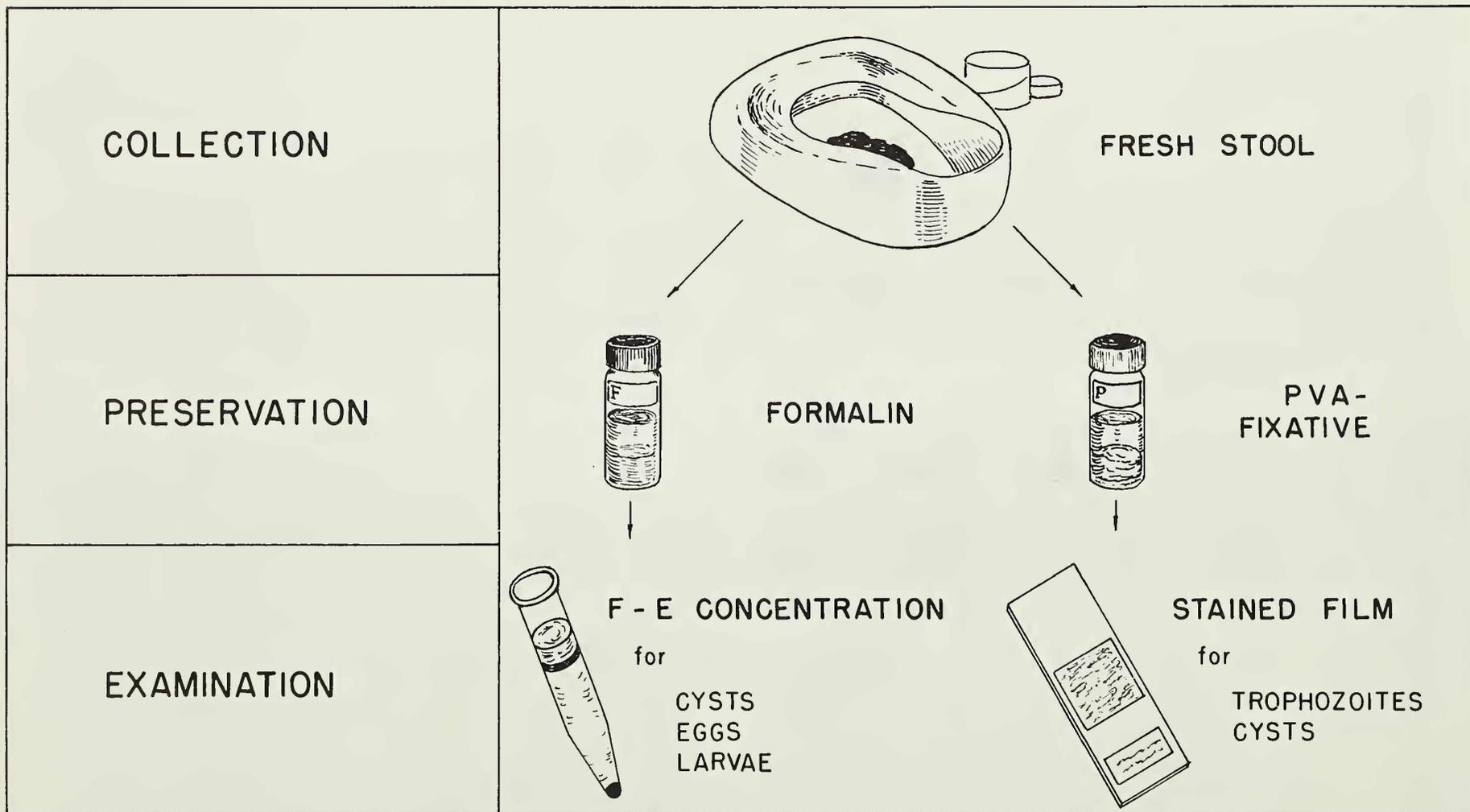
COLLECTION — FRESH STOOL

PRESERVATION — FORMALIN — PVA-FIXATIVE

EXAMINATION — F-E CONCENTRATION
for
CYSTS
EGGS
LARVAE

STAINED FILM
for
TROPHOZOITES
CYSTS

c. Collection of multiple specimens with and without catharsis

Because of the intermittent passing of parasites from the host, examination of multiple specimens is desirable. *Ascaris,* hookworm, and *Trichuris* eggs appear almost daily in feces. Cysts of *Entamoeba histolytica* and eggs of certain of the helminths such as *Schistosoma* species and *Diphyllobothrium latum* are passed intermittently. These irregularities emphasize the need for collection of at least three specimens spread over 10 to 14 days.

Normally, passed stool specimens spaced several days apart are preferable to specimens obtained by catharsis or sigmoidoscopy, since cysts are more likely to be present in passed stool specimens. Purged specimens increase the possibility of finding organisms. A cathartic of sodium sulphate or buffered phospho-soda is preferable to magnesium sulphate since such cathartics affect the morphology of the organism less. Each bowel movement should be collected separately, numbered serially, and delivered promptly to the laboratory. Egg, larvae, cysts, and trophozoites may be found in such specimens. If examination of the specimen is to be delayed, add a portion to PVA fixative.

d. Collection of specimens by sigmoidoscopy

In amebiasis, if stools are negative, material may be obtained by sigmoidoscopy immediately following a normal bowel movement, or if a cathartic is given, after a lapse of 2 to 3 hours. Collect the specimens with a serologic pipette rather than a cotton swab by aspirating material from any visible lesion and the mucosa. Pathologic areas or the mucosa wall may also be gently curetted. Examination of sigmoidoscopic specimens for diagnosis of amebiasis must be immediate, but after the direct examination, PVA fixative may be added and the preparation dried and stained.

e. Collection of specimens other than feces

Sputum specimens as well as stools should be collected in suspected cases of paragonimiasis. Pulmonary amebiasis and echinococcosis may also be diagnosed by sputum examination. Urine specimens are used in the diagnosis of *Trichomonas vaginalis* and *Schistosoma haematobium.* The optimum urine specimen to be examined for the latter is one passed at or shortly after noon. Vaginal swabs or scrapings are used in diagnosis of *T. vaginalis.** Anal swabs or cellulose tape specimens are the usual means of collecting the eggs of *Enterobius vermicularis,* as shown in Figure 3. Specimens taken between 10 p.m. and 12 midnight, or in the early morning before defecation, are best. Three consecutive examinations are desirable, and a short delay in examination of either the swab or tape specimen makes no particular difference, but the specimens should be refrigerated if examination is delayed for more than a day. Biopsied material for the dagnosis of schistosomiasis may be collected from the colon, rectum, or bladder and, for amebiasis, from the rectum or liver. Biopsy is not recommended for echinococcosis because of the danger of complications resulting from the leaking of hydatid fluid into the surrounding tissue. Duodenal drainage often reveals organisms when stool specimens are negative for *Strongyloides stercoralis* and *Giardia lamblia* and should be collected when diagnosis cannot be established by fecal examination.

Biopsy material (human gastrocnemius or deltoid muscle) may be collected for diagnosis of trichinosis. The muscle may be fixed and sectioned or the fixed tissue may be submitted for examination and sectioning and staining. Serologic methods are more commonly used in the diagnosis of trichinosis than direct examination of tissue for the larvae. Animal biopsy or autopsy meat or other foodstuffs of animal origin suspected of harboring *T. spiralis* larvae should be submitted to the laboratory in plastic bags and liberally covered with sodium borate powder. *The specimen should not be frozen.* The borate preserved meat may then be subjected to pepsin-HCl digestion for recovery of larvae.

* The swabs may be examined as a wet mount or, preferably, they may be placed in a suitable culture medium.

Figure 3
USE OF CELLULOSE-TAPE SLIDE PREPARATION FOR DIAGNOSIS OF PINWORM INFECTIONS
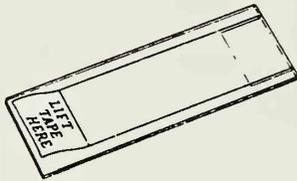
a. Cellulose-tape slide preparation
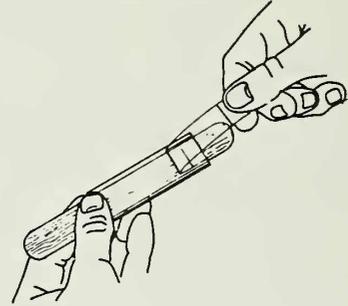
b. Hold slide against tongue depressor one inch from end and lift long portion of tape from slide
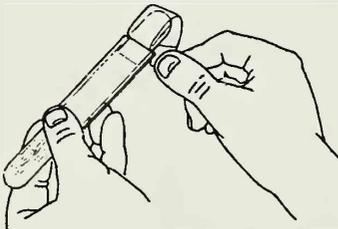
c. Loop tape over end of depressor to expose gummed surface
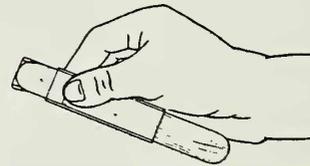
d. Hold tape and slide against tongue depressor

e. Press gummed surfaces against several areas of perianal region
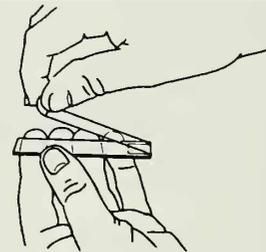
f. Replace tape on slide
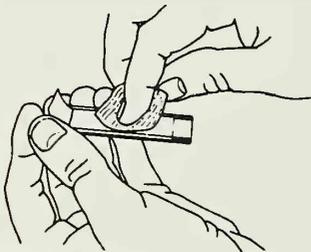
g. Smooth tape with cotton or gauze

Note: Specimens are best obtained a few hours after the person has retired, perhaps at 10 or 11 P.M., or the first thing in the morning before a bowel movement or both.

Skin snips are the specimens of choice in infections with *Onchocerca volvulus* as the microfilariae do not appear in the blood stream. Tissue fluid obtained by puncture and aspiration of a nodule may also show organisms.

f. Serum

Immunodiagnostic tests for intestinal parasite infection are considered to be useful routine diagnostic procedures in only a few diseases, as shown in Table 2.

ARTHROPOD-BORNE DISEASES (SPECIMENS FOR IDENTIFICATION OF ARTHROPODS)

Arthropod ectoparasites play an important role in the transmission of plague, typhus, tularemia, spotted fever, relapsing fever, and several other diseases. Any specimen submitted should be divided, one portion to be used for identification and the other for isolation of any virus or rickettsia that may be present. The sample for identification should be preserved in, preferably, 70% alcohol or in 2% formalin. Samples for isolation should be collected alive and stored on dry ice until tested. Table 3 shows the method of preserving and handling insects and other arthropods according to the kind of examination to be made.

TABLE 3

Preserving and Handling Insects and Other Arthropods

| For Identification	
Moist	Dry
In 70% alcohol or 2% formalin	Between layers of tissue or lens paper. Not cotton
Ticks	Mosquitoes
Mites	Flies
Fleas	True bugs
Lice	Wasps, bees
Fly maggots	Moths
Spiders	Butterflies
Mosquito larvae	
Small flies	
Bed bugs	
Ants	
Caterpillars	

For Demonstration of Microorganisms or Antibodies
Suspend in bottle of 1% sodium chloride solution.

Ectoparasites usually are wingless arthropods. Ticks and mites have eight legs in the nymphal and adult stages. Fleas and lice are true insects with six legs. Fleas are very active jumping insects, compressed from side to side, with elongated legs adapted for jumping. Lice are more sluggish insects, depressed from top to bottom, with legs adapted for grasping hairs. Illustrations of various kinds of arthropods are shown in Figure 4.

1. *Ectoparasites.* Ticks, mites, fleas, and lice should be collected from bedding, animal burrows, or birds' nests with fine forceps, aspirators, or applicator sticks. They may be simply combed, brushed, or knocked off an infected animal.

2. *Flies and Mosquitoes.* These arthropods may be collected with insect nets, aspirators, chloroform tubes, cyanide jars, light, bait, or fly traps. Barns, outbuildings, the under surfaces of bridges, or other adult resting places are the best areas in which to find these insects. Tubes for picking up live insects, holding cages, storage boxes, and bottles are illustrated in Figure 5. All specimens should be submitted unmounted, and large numbers of specimens may be mailed in pill boxes or similar containers. Specimens may be mailed wrapped between layers of lens paper or cleansing tissue with a layer of absorbent cotton next to the top and bottom of the box to absorb vibrations and prevent breakage. *Do not ship mosquitoes or flies between layers of absorbent cotton.* If specimens are mounted for identification, they should be fastened with clear fingernail polish or liquid cement to an insect point or an insect pin. Larger flies may be pinned through the thorax. *Poorly mounted specimens are worse than those unmounted.*

3. *Arthropod specimens submitted for virus or rickettsia isolation.* For this purpose it is of the utmost importance that ticks, mites, fleas, lice, mosquitoes, or other insects be collected alive, sealed in ampules or rubber-stoppered test tubes, and stored on dry ice until tested. If dry ice is not available, temporary storage in a mechanical deep freeze chest is satisfactory. *Collection or storage in chloroform tubes or cyanide jars is not permissible* because these materials inactivate many viral and rickettsial agents.

Figure 4
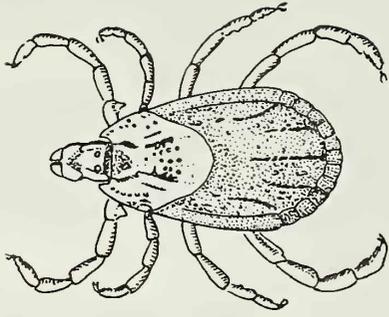
Dermacentor andersoni - Rocky Mountain Wood Tick
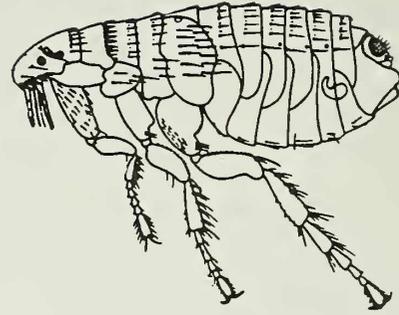
Ctenocephalides felis - Cat Flea
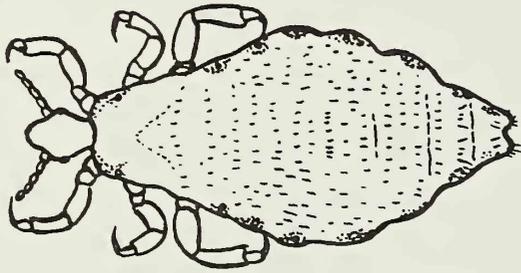
Pediculus humanus - Human Head and Body Louse
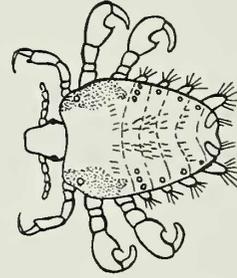
Pthirus pubis - Human Crab Louse
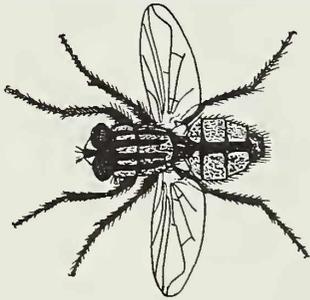
Musca domestica - House Fly
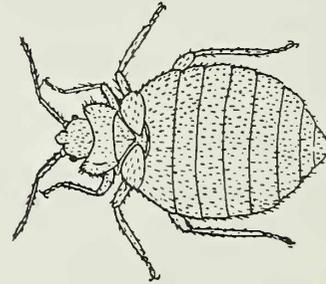
Cimex lectularius - Bed Bug
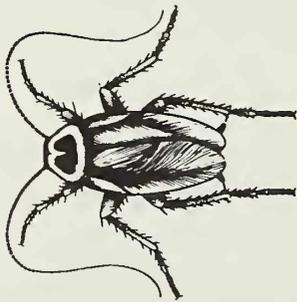
Periplaneta americana - American Cockroach
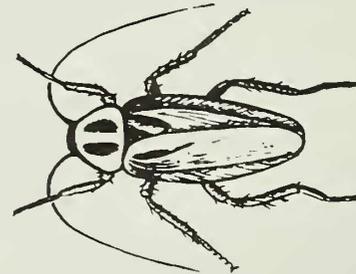
Blatella germanica - German Cockroach
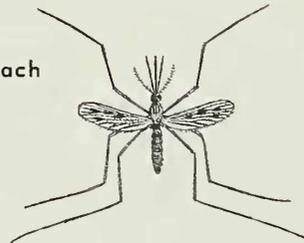
Anopheles quadrimaculatus - Malaria Mosquito

28

Figure 5
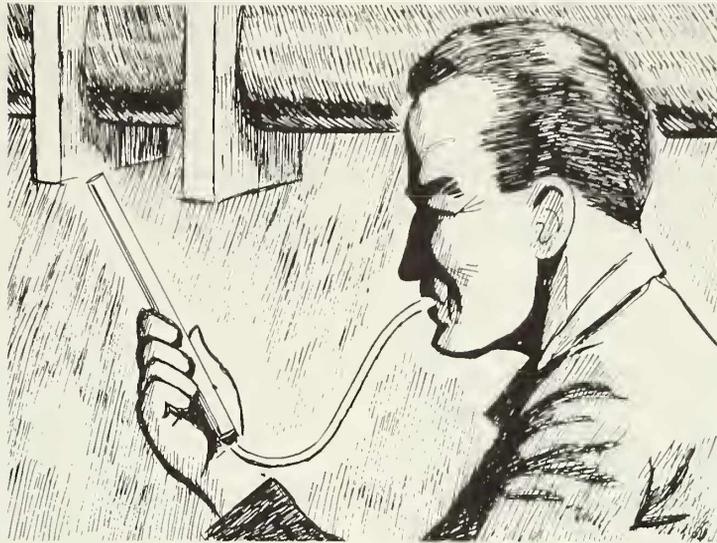
Collection of insects alive with an aspirator, using breath to supply the suction.
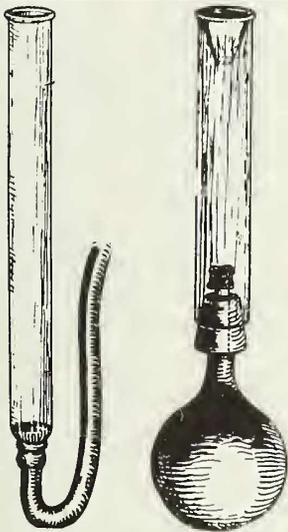
Tubes for picking up insects with an aspirator.

Bottle of saline (1% Na Cl) for specimens to be examined for microorganisms of antibodies. Formalin (10% = 3.7% formaldehyde) may also be used for storage of specimens.
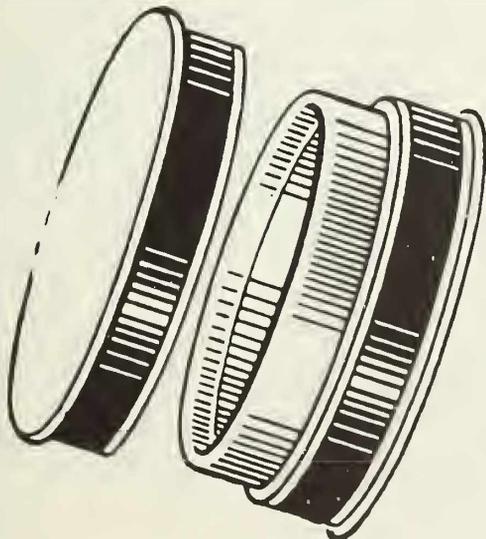
Pill box for storage of dead insects.

Holding cage for insects made from a tin can with perforated lid kept humid by a wet cloth thrown over the cage during transit.

29

VIRAL AND RICKETTSIAL DISEASES

Diagnosis of viral and rickettsial diseases in the laboratory may be attempted by three general procedures.

1. Isolation and identification of the inciting agents;
2. Demonstration of a rise in titer of specific antibodies during the course of the illness;
3. Examination of the infected tissues for pathologic alterations.

It is rarely possible or necessary to use all three procedures in diagnostic work. A decision as to which procedure should be followed is dictated by the nature of the infection, the stage of the illness when the patient is first seen, and the amount of information the methods will yield in relation to the time, effort, and expense involved.

Direct microscopic methods are rarely used and are of limited usefulness except in rabies diagnosis and a few other special cases. Aside from immunofluorescence, microscopy is usually confirmed by some other procedure such as serology or isolation of the agent.

Serologic tests have far greater usefulness in the diagnostic laboratory than either of the other approaches. Serologic methods generally yield information more rapidly and less expensively than isolation and identification of an agent, but they do not give the same degree of assurance of etiologic involvement. In fatal infections, when there has been insufficient time for specific antibody development, isolation of the causative agent is the only means of diagnosis. When the virus exists in many antigenic types, as in the Coxsackie group, it is essential to isolate the virus from clinical material. Serologic tests are possible only for those diseases whose causative agents have been isolated and from which satisfactory antigens can be produced. Obviously, some exception must be made for those diseases in which a biologically nonspecific phenomenon can be useful in suggesting a diagnosis, as in the Weil-Felix reaction for certain rickettsioses, and the heterophile test for infectious mononucleosis. Serologic methods alone with contribute little to establishing the *etiology* of diseases of unknown causation.

The types of specimens that may be submitted for laboratory examination in viral and rickettsial diseases are more varied than in any other type of infection. Depending on the disease, the specimens include: nasal swabs, throat swabs, swabs of oral lesions, saliva, sputum, nasopharyngeal swabs, nose and throat washings, stool or rectal swabs, cerebrospinal fluid, vesicle fluid, pleural effusion fluid, bubo aspiration fluid, muscle biopsy, crusts, scrapings from fever sores, postmortem tissue and paired sera. In interpreting isolation results, it should be remembered that the mere presence of a virus in excreta does not necessarily establish the etiological relationship of the agent to the disease. Inapparent and mixed infections, particularly with enteroviruses, are common and the etiologically unrelated virus may even give rise to antibody formation which coincides with the timetable of the disease.

1. *Preparation and Shipment of Diagnostic Specimens*
   a. **Type of Specimen to be Collected**
      (1) Virus isolation. Materials for isolation must be freshly obtained and, if possible, collected with aseptic precautions. Source of materials depends on the type of clinical disease (that is, throat swabs or throat washings in respiratory infections, etc.). Usual materials include blood, throat washings, sputum, feces, effusion fluid, tissue biopsies, autopsy tissue, or lesion scrapings. *No preservative or fixative should be added.* After collection, blood for virus isolation should be frozen without further treatment.
      (2) Serological tests. Serum should be obtained from clotted blood collected and kept under sterile conditions. *No preservative need be added.* Separating the serum from the clot at the source prevents hemolysis which interferes with some tests. At least 10 ml. of blood (5 ml. of serum) and preferably 20 ml. of blood should be collected for serological tests to permit a multiplicity of tests, confirmation, or repetition of equivocal results when necessary.
   b. **Time to Collect Specimens**
      (1) Virus isolation. The time in the course of the clinical disease that specimens are collected is of utmost importance. In general, for isolation techniques the earlier in the acute stage the specimen is taken, the better the chance for successful isolation. The specimen should certainly be taken while the patient is still acutely ill and febrile.
      (2) Serological tests. Except in outbreaks of encephalitis, serum specimens *must be paired* since only a *rise* in specific

antibody titer is positive evidence of current infection with the responsible agent. The first sample should be collected as early in the illness as possible and the second from 2 to 4 weeks after the beginning of convalescence. Even in arbovirus infections antibody in the the first serum is only suggestive of recent infection and must be followed up with a second serum.

c. **Container for Specimen**

(1) Virus isolation. Materials for virus isolation must be frozen if it will take longer than a few hours to get them to the laboratory. This requires dry ice. Dry ice releases CO_2 gas which is deleterious to most viruses; therefore these specimens must be completely sealed off (flame-sealed ampules are best for fluid specimens). With samples placed in bottles closed with tightly fitting rubber stoppers, the tops should be sealed with at least three turns of high quality waterproof adhesive tape. If facilities for freezing stool specimens are not available, they can be emulsified in an equal volume of 1 molar magnesium chloride and shipped in an insulated container with cans of frozen water (picnic cooler type) and packed in wood shavings.

(2) Serological tests. The container must protect the specimen from loss by breakage as well as from contamination and deterioration. Serum samples in test tubes with tight rubber stoppers or, preferably, sleeve stoppers (reinforced by adhesive tape) are satisfactory. Cork stoppers are quite unsatisfactory, and the type of screw-cap vials frequently loosen and leakage occurs.

d. **Packing and Shipping of Specimens**

(1) Virus isolation. Virus isolation samples once ampuled should be quickly frozen (on dry ice) and maintained in the frozen state (preferably at $-70°C$.) until the time for testing. For safety purposes and to prevent breakage, each specimen tube should be individually wrapped in either paper towels, facial tissues, or other absorbent padding material. One or more padded

specimens can be placed in a small cylindrical shipping container which is packed directly against 5 to 10 pounds of dry ice (the amount of dry ice depending upon the distance to be shipped) in the center of a box large enough for 6 inches of insulating material (paper, cotton, etc.) to surround it. Shipment should be by the most rapid means available (usually air mail, special delivery). The laboratory should be notified by wire of the contents, mode of shipment, air express way-bill number, and time of expected arrival. Whenever possible, specimens should be shipped so that they will arrive at the laboratory during the working day and not on weekends.

Birds submitted for psittacosis studies should be thoroughly soaked in disinfectant, placed in a plastic bag, and then packed in a shipping box with dry ice. If rapid delivery is possible, cans of frozen water may be used.

(2) Serological tests. If adequately protected against spillage and breakage, serum samples for serological tests may be shipped without refrigeration in ordinary mailing containers. However, these specimens should also be sent by the most rapid method.

2. *Minimum Data to be Supplied With Specimen*

Name of Patient.

Age.

Summary of pertinent history *including date of onset,* physical findings, and clinical laboratory tests.

Virus group suspected.

Type of material submitted and *date of collection.*

Indication of other similar cases in family or vicinity.

Viral or rickettsial vaccines given to patient and dates administered.

Exposure to animals or insect vectors.

Antibiotic treatment

All specimens should be forwarded to the State public health laboratory, accompanied by Form PHS 3.332 (see Appendix V). These forms are available at your State laboratory and should be filled in as completely as possible in order to expedite the processing of your specimen.

31

3. *Laboratory Methods in Diagnosis*
   a. Direct Methods:
      (1) Preparation of specimen for inoculation. Certain types of materials, such as throat washings, sputum, and feces, must be freed of contaminating bacteria and molds before they are used for inoculation. Ultra-centrifugation can separate the contaminants at one speed and concentrate the virus at another. Filtration, chemical treatment, and especially the addition of antibiotics are also useful in removing contaminants. An effort should be made however, to collect materials as free from contaminants as possible.
      (2) Animal inoculation methods. Various species of animals are inoculated by different methods (intracerebral, intraperitoneal, intranasal, intravenous, etc.), depending on the type of human disease being studied and the type of material available. Symptoms and pathological responses are then observed. These methods are used in the isolation of the agents of rabies, psittacosis, lymphogranuloma venereum, encephalitis, herpes, rickettsial, and other diseases.
      (3) Chick embryo inoculation methods. Embryonic tissue is more susceptible to invasion by most viruses than adult tissue. Chick embryos of varying ages are inoculated by different routes (yolk sac, allantoic sac, amniotic sac, etc.) and are observed for death, for specific pathological lesions, or as a source of antigenic material for specific serological identification against known positive immune sera (used in influenza, smallpox, mumps, etc.).
      (4) Tissue culture methods. Tissue culture represents the use of specific tissues in culture where extracellular factors of the media can be controlled. This method has become the laboratory procedure of choice in the study of many viruses (those of poliomyelitis, influenza, chickenpox, etc.) for isolation or for antibody determination and measurement.
   b. Indirect Methods:
      (1) Examination of tissues for pathological change. Certain viruses cause specific cellular responses characterized by inclusion bodies, such as Negri bodies in rabies, which are diagnostic of active infection. Some of the larger viruses—lymphogranuloma venereum, for example can actually be seen in tissue smears as elementary bodies. Fluorescent antibody techniques have recently been applied to the direct examination of infected tissues or exudates for the rapid identification of specific viral antigen. This technique has been most widely used in the diagnosis of rabies.
      (2) Complement fixation tests. Specific antigens are available for testing *paired* acute and convalescent sera in many viral and rickettsial diseases. In rickettsial diseases, the complement fixation test is more specific than the Weil-Felix proteus agglutination procedure.
      (3) Red cell agglutination tests. Certain viruses cause red blood cells to agglutinate. The presence of specific antibodies against that virus will inhibit the agglutination. This type of test is frequently used in influenza, mumps, Newcastle virus disease, certain encephalitis infections, and rubella.
      (4) Neutralization tests. In the neutralization test, the fact that a given virus has been inactivated or "neutralized" by specific antibody present in a serum specimen is demonstrated by the failure of a susceptible animal or tissue culture to become infected when inoculated with the serum-virus mixture. This procedure can be used with *paired* acute and convalescent sera in any virus or rickettsial disease for which there is a susceptible animal or an appropriate tissue culture system in which the etiologic agent will multiply.

4. *Interpretation of Laboratory Results*
   a. Virus Isolation:
      Failure to isolate a specific virus from a specimen does not rule out that agent, or an unsuspected one, as the possible cause of the illness in question. When a virus is isolated, it signifies current *infection* (which

may be silent) with that virus. It usually, but not always, means that the current apparent *illness* is due to that virus. The results of virus isolation attempts must be correlated with the clinical, epidemiologic, and serologic data.

b. Serologic Tests:

Since it has been found that some individuals already have antibodies against certain viruses because of previous contact with that virus, the finding of antibodies in a single serum sample taken during or after a particular illness does not prove the etiology of that illness. However, a definite (4-fold or higher) rise in antibody titer from the acute stage of the disease to convalescence is usually significant. Although serologic tests yield indirect evidence of virus activity, they are more frequently informative than virus isolation attempts. An effort should be made in *all* cases to submit *paired* serum specimens in order to make a presumptive serologic diagnosis or to confirm the significance of the virus isolation by demonstrating a rise in antibody titer against the virus dur-the course of illness.

Because of the large numbers of antigenic types, serologic studies on paired serum specimens usually cannot be performed in suspected enterovirus infections unless a virus is isolated from clinical material. When pericarditis or pleurodynia is present and adequate clinical information is provided, the microneutralization test for Coxsackie B viruses can be performed.

In certain situations. a third serum specimen (taken late in convalescence, 2 or 3 months after onset) is extremely valuable. This is a recognized necessity in lymphocytic choriomeningitis and most rickettsial diseases. In lymphocytic choriomeningitis, complement fixing antibodies may not occur until late in the course of the disease, approximately in the eighth week. In rickettsial diseases and in psittacosis and lymphogranuloma venereum, the production of complement fixing antibodies may be suppressed or delayed in patients who are treated early with broad spectrum antibiotics.

5. *Packing and Shipment of the Specimens for Rabies Diagnosis*

After decapitation of the animal in the field, the head should be promptly cooled down and kept cold. Whenever possible, it should be delivered by messenger. If no messenger service is available, the head should be packed for shipment by the fastest common carrier. It should be put into a suitable watertight metal container and tightly sealed. This container. in turn should be put into a larger watertight metal container. Cracked ice should be packed between the inner and outer container. The package should be clearly labelled and shipped to the laboratory with utmost dispatch.

NOTE: Although freezing the specimen and shipping it frozen in dry ice (solid carbon dioxide) or in nitrogen flasks will preserve the virus, quick microscopic examination may be delayed because of the time necessary for the head to thaw. Frozen portions of brain and salivary glands are easier to handle in the laboratory than are frozen entire heads. Immediately upon thawing, the tissues should be prepared for direct microscopic examination for Negri bodies, for the fluorescent antibody test, or for the mouse inoculation test.

33

The following information is desirable when animal heads are received for examination: the species and breed of the animal; whether it was in contact with other animals; whether the animal died or was killed, and, if the latter, the means used in destroying it; whether the animal was confined and observed for an appropriate time before death, and, if so, for how long; symptoms of rabies, if any; and history of vaccination against rabies.

TABLE 4

Diagnostic Procedures for Viral and Rickettsial Diseases

Disease Syndrome	Associated Agent	Serological Tests				Serum Specimens Required[1]		Specimens for Isolation
		CF	Neut.	HI	FA	Acute	Convalescent	
CENTRAL NERVOUS SYSTEM								
	Arthropod-Borne Enc.[2]							
Encephalitis, Meningoencephalitis, Aseptic Meningitis, Viral Meningitis	Eastern Equine[2]	X	X	X		Immediately After Onset	2-4 weeks later	Blood and/or Brain
	Western Equine[2]	X	X	X		"	"	"
	St. Louis[2]	X	X	X		"	"	"
	Venezuelan Equine	X	X	X		"	"	"
	California	X	X	X		"	"	"
	Other Arboviruses		X	X		"	"	Blood
	Poliovirus	X	X			"	"	Feces, throat swabs, tissue
	Enterovirus		X			"	"	Spinal fluid, feces
	Lymphocytic choriomeningitis	X	X			"	"	Blood, Brain
	Rabies		X		X	"	"	Brain
	Herpes Simplex	X	X	X		"	"	Brain, Vesicular fluid
OTHER ARBOVIRUS-ASSOCIATED DISEASE								
Colorado Tick Fever		X	X			Immediately	2-4 weeks later	Blood
Dengue-Type		X	X	X		"	"	Blood
Hemorrhagic Disease		X	X	X		"	"	Blood
Vesicular Stomatitis		X	X			"	"	Vesicular fluid, blood
Yellow Fever		X	X	X		"	"	Blood
RICKETTSIAL INFECTIONS								
Rocky Mountain Spotted Fever		X				Immediately	2-4 weeks later	Blood
Typhus, murine and epidemic		X				"	"	Blood
Rickettsialpox		X				"	"	Blood
Q Fever		X				"	"	Blood and Sputum

34

Disease	Agent					Acute sera	Convalescent sera	Specimen
Herpangina[3]	Coxsackie A group		X			Immediately	2-4 weeks later	Feces, throat swab
Pleurodynia[3]	Coxsackie B group		X			"	" "	" " "
Benign pericarditis[3]	" " "		X			"	" "	" " "
Myocarditis[3]	" " "		X			"	" "	" " "
Hand, foot, mouth syndrome	Coxsackie A16		X			"	" "	Vesicular fluid, throat, stool

RESPIRATORY SYSTEM

Disease	Agent					Acute sera	Convalescent sera	Specimen
Influenza	Influenza Group	X	X	X		Immediately After Onset	2-4 weeks later	Throat swab
Acute Respiratory Disease and Pneumonia	Adenovirus Group	X	X	X		"	" "	" "
	Parainfluenza	X	X	X		"	" "	" "
	Respiratory syncytial	X	X			"	" "	" "
	Psittacosis	X				"	" "	" " , sputum[4]
	Mycoplasma p'moniae	X	X	X		"	" "	" "

VESICULAR DISEASES

Disease	Agent					Acute sera	Convalescent sera	Specimen
Vesicular Eruptions	Variola-Vaccinia	X	X		X	"	" "	Vesicular fluid, lesion scrapings, crusts
	Chickenpox-H. Zoster	X			X	"	" "	
	Herpes Simplex	X	X			"	" "	

MISCELLANEOUS

Disease	Agent					Acute sera	Convalescent sera	Specimen
Mumps		X		X		Immediately	2-4 weeks later	Throat swab and blood
Measles		X		X		"	" "	" " "
Rubella (German Measles)		X	X	X	X	"	" "	" " "
Epidemic Keratoconjunctivitis	Adenovirus	X	X	X		"	" "	Conjunctival scrapings
Lymphogranuloma venereum		X				"	" "	
Cytomegalic Inclusion Disease	Cytomegalovirus	X				"	30 days later	Urine, throat swab

[1] Paired sera are mandatory to determine whether antibodies have appeared or increased in titer during illness.

[2] In suspected arbovirus epidemics single acute sera are helpful in early detection of infection, but paired specimens are still needed to confirm the infection in an individual.

[3] Serologic tests are performed only in well documented cases of myocarditis and pericarditis or when a virus is isolated from clinical material.

[4] See page —— for instructions on shipping birds.

[5] Conjunctival smears are examined for inclusion bodies.

35

APPENDIX I

PART 124
NONMAILABLE MATTER

124.1 INTRODUCTION

.11 DESCRIPTION. Nonmailable matter includes all matter which is by law, regulation, or treaty stipulation prohibited from being sent in the mail or which cannot be forwarded to its destination because of illegible, incorrect, or insufficient address.

.12 APPLICABILITY. The harmful or objectionable things identified or described in this part are some of the matter which may not be sent through the mail, as a matter of absolute prohibition. See part 125 for matter mailable only under special rules or conditions. Notwithstanding any statement contained in part 124, which covers only some of the items prohibited in the mail, the burden rests with the mailer to assure that he has complied with the law. In addition to the nonmailable items mentioned in this part, certain other articles are prohibited in the mail to military post offices overseas (part 127).

.13 PENALTIES FOR VIOLATION. Severe penalties, by fine or imprisonment, or both, are provided for persons who knowingly mail or cause to be mailed, any matter which has been declared nonmailable under law.

.14 NONCONFORMITY WITH POSTAL REGULATIONS. Regardless of its nature, matter may not be mailed in any form if done in violation of postal regulations for such reasons as failure to pay postage, improper size or weight, improper permits, improper addresses, etc.

.15 RESPONSIBILITY OF MAILER. When mailers are in doubt as to whether any matter is properly mailable, they should ask the postmaster. Even though the Post Office Department has not expressly declared any matter to be nonmailable, the mailer of such matter may be held fully liable for violation of law if he does actually send nonmailable matter through the mail.

124.2 HARMFUL MATTER

.21 GENERAL PROVISIONS OF LAW

Any articles, compositions, or materials, which may kill or injure another, or injure the mail or other property, are nonmailable. This includes but is not limited to:

a. All kinds of poison or matter containing poison.

b. All poisonous animals, except scorpions (see 125.35), all poisonous insects, all poisonous reptiles, and all kinds of snakes.

c. All disease germs or scabs.

d. All explosives, flammable material, infernal machines, and mechanical, chemical, or other devices or compositions which may ignite or explode.

* * *

PART 125
MATTER MAILABLE UNDER SPECIAL RULES

125.1 LEGAL RESTRICTIONS

.11 HARMFUL MATTER

.111 Certain items barred from the mail, as set forth in part 124, may be mailed if prepared and packaged in accordance with this part. These are items not outwardly or of their own force dangerous or injurious to life, health, or property.

.112 This part covers generally some of the more common situations; however, the burden rests with the mailer to assure that he has complied with the law and that anything shipped by him has been properly prepared and packaged. The ordinary test of adequate preparation and packaging is whether the

36

contents of a parcel are safely preserved under ordinary hazards of mail handling and transportation.

.113 Products, materials, and devices are created or modified with such frequency that the Post Office Department is unable to issue general rulings in advance to govern adequate preparation and packaging. Any mailer may, however, request the Post Office Department, in advance, for a specific ruling as to mailability of his item. The request should be addressed to the local postmaster, who will forward it to the Classification and Special Services Division, Bureau of Operations, Washington, D.C. 20260.

.12 APPLICABILITY OF OTHER LAWS

* * *

.122 Any special conditions or limitations placed on transportation or movement of certain things shall govern admissibility to the United States mail, when imposed under law by the U. S. Department of the Treasury; U. S. Department of Agriculture; U. S. Department of Commerce; U. S. Department of Health, Education, and Welfare; Interstate Commerce Commission; or any other Federal department or agency having legal jurisdiction.

.13 PENALTIES

Severe penalties of fine or imprisonment, or both, are provided by law, for anyone who knowingly deposits for mailing or delivery, or causes to be mailed or delivered, anything declared nonmailable under law. Failure to comply with the regulations of the Postmaster General, as prescribed in this part, as to matter otherwise nonmailable, constitutes a violation of law.

125.2 CONDITIONS FOR MAILING

.21 GENERAL NATURE OF PRECAUTIONS REQUIRED

.211 The restrictions against mailing of harmful matter, from which relief is granted by this part, are intended to prevent damage or harm to postal and transportation personnel, to prevent damage or destruction of other mail and of property, to avoid obnoxious odors, and to prevent the spread of disease and infection. Special preparation and packaging are required to protect against such contingencies.

.212 Basic precautions, covered generally in this section, relate to the inner containers holding the harmful matter, internal cushioning and protection, and exterior packaging and marking.

.22 LIQUIDS (NONFLAMMABLE) AND POWDERS

.221 Precautions shall be taken in the case of liquids, pastes, salves, ink powders, pepper, snuff, or other pulverized materials against damage to mail and property from leakage and against caustic, irritant, toxic, or soiling effect on mail handling personnel.

.222 Containers shall meet any applicable Interstate Commerce Commission or other Federal speci-

fications. Closures must effectively seal the contents against leakage. Friction tops must be fastened with solder, clips, or otherwise so that they will not come off under impact.

.223 Glass or other breakable containers of liquid must be packaged to withstand handling enroute. The container shall be cushioned inside the carton to absorb shock and impact. Where feasible, absorbent material shall be used, to take up all the liquid in case of breakage.

* * *

125.7 IDENTIFICATION AND MARKING

.71 IDENTIFICATION OF CONTENTS. The identity or nature of contents of anything mailed under any of the provisions of part 125 shall be stated plainly on the outside of the parcel, as a condition of mailing.

.72 IDENTIFICATION OF MAILER AND ADDRESSEE. The full name and address of both the mailer and the addressee shall be written in ink, rubberstamped, or pasted on the outside of any package whose mailing is covered by part 125.

.73 LABELS. Any labels required under Federal law or under any regulations issued by any Federal agencies pursuant to Federal law shall be pasted to the outside of the parcel.

* * *

PART 221
CONDITIONS APPLICABLE TO ALL CLASSES

221.1 PREPARING AND ADDRESSING

.11 PREPARING

.111 Senders must prepare articles securely, especially if they are for distant countries. International mail is handled more often and subjected to greater pressure and friction than domestic mail, hence it must be enclosed in strong envelopes or other wrappings.

* * *

.114 Articles other than letters and letter packages (AO mail) must be prepared in such a way that their contents are sufficiently protected but so as not to hinder quick and easy inspection of the contents. They should be placed under wrapper, on a roller, or between cardboard; in open bags, boxes, envelopes, or containers or in closed, unsealed bags, boxes, envelopes, or containers provided with fasteners that can be easily opened and reclosed without being dangerous; or they may be tied with string or twine in a manner that will permit them to be easily untied. Sealing of postal union *other articles* is not permitted, even if registered, and they must be prepared in such a way that other articles do not run the risk of being trapped by them.

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.325 *Perishable Biological Materials.* Perishable biological materials, including those of pathogenic nature, when sent in the postal union mail are accepted only as LETTER PACKAGES. The following conditions apply:

a. *Mailing Restrictions*

If a country prohibits perishable biological materials this is shown under *Prohibitions* in the country item in the Directory of International Mail. The packages must be packed as prescribed in 221.325c and must bear distinctive violet labels by which they can be readily recognized and receive careful handling and prompt delivery.

b. *Qualification of Mailers*

(1) Only officially recognized laboratories may send or receive letter packages containing perishable biological materials. Laboratories of the following categories are so designated:

Laboratories of local, State, and Federal government agencies.

Laboratories of federally licensed manufacturers of biologic substances derived from bacteria and viruses.

Laboratories affiliated with or operated by hospitals, universities, research facilities, and other teaching institutions.

Private laboratories licensed, certified, recognized, or approved by a public authority.

(2) A laboratory desiring to mail letter packages containing materials of this kind shall make written application on its letterhead stationery to the Classification and Special Services Division, Bureau of Operations, Post Office Department, Washington, D.C. 20260, explaining its qualifications and those of the prospective addressee to send and receive such materials, and stating how many packages are to be mailed. On approval, the mailer will receive a sufficient number of the violet labels for the contemplated shipments.

c. Packaging

(1) Perishable biological material not of a pathogenic nature must be packed in a nonporous container surrounded by sufficient absorbent material to take up all the liquid and must be placed in an outer protective container where it should fit tightly to avoid any shifting.

(2) Perishable biological material of a pathogenic nature must be packed in a tightly closed bottle or tube or heavy glass wrapped in thick absorbent material rolled several times around the bottle or tube and tied at the ends, sufficient in quantity to absorb all the liquid; the wrapped container must be placed in a strong well-closed metal box constructed to prevent any contamination outside of it. This metal box must be wrapped in cushioning material and placed in an outer protective box where it should fit tightly to avoid shifting. The outer container must consist of a hollow block of strong wood, metal, or other equally strong material with a tight lid so fitted that it cannot open during transportation.

(3) In addition to the requirements in (1) and (2), packages must comply with the regulations governing the transmission of such materials in the domestic mail.

(4) The mailer must place on each package one of the violet labels mentioned in a and b(2).
* * *

APPENDIX II

Selected Transport Media and Reagents

Some biological specimens will withstand shipment better if placed in a protective medium or if a preservative is added. Likewise, isolated cultures of bacteria, fungi, or viruses should be placed in or upon a medium which will enhance or preserve their viability in transit. It this appendix no attempt is made to list all suitable transport media. The sender should give careful consideration to the selection of media in each specific case. Several "transport" media are available commercially.

Some selected media recommended by the NCDC are:

1. **Merthiolate solution for preserving blood serum**

   1.4 gm. sodium borate (borax)

   1.0 mg. merthiolate

   100.0 ml. H_2O

   Dissolve the sodium borate in the water first and then add the merthiolate. Use 0.1 ml. of the above solution per 10 ml. of serum to give 1:10,000.

2. **Sabouraud - cycloheximide - chloramphenicol agar* for selective isolation of pathogenic fungi.**

   Composition: Sabouraud dextrose agar (2% agar content)
   Cycloheximide** 0.5 mg./ml.
   Chloramphenicol*** 0.05 mg./ml.

   Preparation: 1 liter
   a. Suspend 65 gm. dehydrated Sabouraud dextrose agar and 5.0 gm. agar in 1,000 ml. distilled water. Heat to boiling.
   b. Add chloramphenicol (50 mg. suspended in 10 ml. of 95% alcohol) to above boiling medium. Remove quickly from heat and mix.
   c. Add cycloheximide solution (500 mg. in 10 ml. of acetone).
   d. Mix well and distribute in tubes.
   e. Autoclave at 118° C. for 10 minutes—no longer. Slant and allow to harden.

3. **Brain heart infusion—cycloheximide-chloramphenicol agar for isolation of fastidious fungi, for example,** *Histoplasma capsulatum* **and** *Blastomyces dermatitidis,* **at 25° C. or room temperature.**

   Composition: Brain heart infusion agar (2% agar content)
   Cycloheximide 0.5 mg./ml.
   Chloramphenicol 0.05 mg./ml.

   Preparation: 1 liter
   a. Suspend 37 gm. dehydrated brain infusion agar and 20.0 gm. agar in 1,000 ml. distilled water. Heat to boiling.
   b. Add chloramphenicol 0.05 mg./ml. to the boiling medium. Remove from heat quickly and mix.
   c. Add cycloheximide 0.5 mg./ml.
   d. Mix well and distribute in tubes.
   e. Autoclave at 118° C. for 10 minutes—no longer. Tube and slant.

4. **Chloramphenicol solution to add to sputum bottles for isolation of** *H. capsulatum*

   Preparation of stock solution.
   Suspend 20 mg. chloramphenicol in 10 ml. 95% alcohol. Add 90 ml. of distilled water. If necessary, heat gently to complete solution. This is a stable solution.

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   * Two essentially similar media are available in dehydrated form: Mycosel agar (BBL, Division of BioQuest); Mycobiotic agar (Difco).

   ** Actidione (The Upjohn Co., Kalamazoo, Mich.)

   *** Chloromycetin (Parke-Davis Co., Detroit, Mich.)

   Use: Add 1.0 ml. of the stock solution to each sterile sputum bottle. This amount is ample for inhibition of contaminants in 1 to 10 ml. sputum. If the solution dries in the bottle before use, its effectiveness is unimpaired. The concentration desired in sputum is approximately 0.2 mg./ml. of chloramphenicol.

5. **Preparation of PVA-fixative for preservation of stool specimens for parasitologic diagnosis**

   Add 6 gm. of polyvinyl alcohol* (PVA) powder to 100 ml. of Schaudinn's fixative at room temperature, stirring constantly.

   Modified Schaudinn's Fixative:
   Glacial Acetic Acid ⸺⸺⸺ 5.0 ml.
   Glycerol ⸺⸺⸺⸺⸺ 1.5 ml.
   Schaudinn's Fixative (2 parts saturated aqueous solution of mercuric chloride and 1 part 95% ethyl alcohol) ⸺⸺93.5 ml.

   Heat to about 75° C. or higher until powder dissolves and suspension clears.

   Cooled to room temperature, the solution should be clear and free of lumps. Solutions prepared with some lots of PVA may remain turbid and may exhibit some precipitate upon cooling. Unless these conditions are excessive, they will not interfere with satisfactory use of the solution. PVA-fixative remains satisfactory for several months and can be used either at room temperature or heated to 50° C.

   A quantity of specimen is thoroughly mixed in a vial containing three or more parts of PVA-fixative. Films for staining can be prepared immediately or months later by spreading two or three drops of the mixture over the surface of a microscope slide. The smear should cover about one-third of the slide surface and it should, to reduce peeling during staining, extend to the edge of the side of the slide. It is important not to have the films too thick and to allow them to dry thoroughly. If the specimen in the vial jells, it can be liquefied by heating in a water bath prior to making the films.

6. **Chopped meat medium.**

Ground meat (fat free)	500 gm.
Distilled water	1000 ml.
1 normal NaOH	25 ml.

   Use lean beef or horse meat. Remove fat and connective tissue before grinding. Mix meat,

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   * Gelvatol—available from Shawinigon Resins Co., Springfield, Mass.

water and NaOH and bring to boil, stirring. Cool, refrigerate overnight and skim off any remaining fat. Filter the mixture through two layers of gauze and spread the meat particles out to partially dry.

Add sufficient distilled water to the filtrate to restore 1 liter original volume and add:

Trypticase or peptone	30 gm.
Yeast extract	5 gm.
Potassium phosphate	5 gm.
Glucose	3 gm.

Adjust pH to 7.8 with 1 N NaOH.

Dispense meat particles in 15 x 125 mm. screw-cap tubes with a small scoop and add the enriched filtrate. Use about 1 part meat particles plus 3 to 4 parts liquid (v/v) per tube. Add a few iron filings to each tube. Tubes should be more than half full (about 8 ml. fluid). Autoclave at 121° C. for 15 minutes.

APPENDIX III

Acceptable Containers for Use in Shipping Specimens

1. Unfrozen specimens
   For shipping small numbers of specimens (1 to 3) of whole blood or serum, any one of the usual double-mailing containers with a metal bottom and metal screw cap is satisfactory, providing the cap is tight. The tubes should be individually wrapped and identified, and the identifying list should be wrapped around the outside of the inner case before this is inserted in the outer case. Sufficient absorbent cotton should be included in the inner case to absorb any liquid resulting from breakage in transit.
   Shipment of large numbers of tubes in a cardboard carton is possible if the tubes are individually wrapped and sufficient absorbent buffer material—cotton, shredded paper, excelsior, etc. —is included between the individual tubes and next to the walls of the carton to absorb shocks and leakage. The conventional cardboard cartons with partitions designed to create a compartment for each tube are most acceptable.

2. Frozen specimens
   Small numbers of frozen sera or tissue blocks may be shipped in metal thermos-type containers or in cartons with dry ice or bags of Snogel or other reversible refrigerant. If the thermos-type container is used, it should be pre-chilled with dry ice for several hours before the specimens are introduced. The specimens should be individually wrapped and identified and packed in such a way that they will not become loose as the dry ice evaporates. Enclose the thermos in an outer carton containing additional dry ice, if necessary, and adequate insulating material to hold the temperature for at least 24 hours longer than the package is expected to be in transit.

Small numbers of frozen specimens may be transported short distances by placing them in insulated paper bags—the so-called "Jiffy Bag" used for ice cream—with a small amount of dry ice. These bags should be enclosed within one or two other "Jiffy Bags" of larger size. Frozen bags of "Snogel" may be used in place of dry ice.
   Larger numbers of tubes should be individually identified and wrapped and packed in cardboard cartons with dry ice above and below and an abundance of insulating material around them. Wrap the carton with several layers of heavy brown wrapping paper and tie or seal it securely with tape. This carton should be enclosed in a larger one which should be wrapped and sealed before shipment. The package should be shipped by the most rapid means available.

3. Refrigerated but not frozen specimens
   Animal heads should always be refrigerated but not frozen during transportation to the laboratory. If the head is that of a small animal, it may be placed in a friction top can of proper size, the lid firmly pressed into position, and the can enclosed in a large size lard can or other container. Cracked ice is used to fill the outer can and, if available, a small amount of dry ice may be included as this will help to hold the temperature down for a longer time without actually freezing the specimen.

4. Glass slides
   Whenever available, slide boxes should be used with tissue paper stuffed between the slides. Otherwise, the slides should be individually wrapped in tissue and shipped in a screw-capped mailing container with suitable absorbent material to prevent breakage. *Under no circumstances, should slides be mailed in a letter or manila envelope.*

APPENDIX IV

Reference Diagnostic Services Available at NCDC

Reference services of the various laboratories of the NCDC are offered to assist the State public health laboratories. Specimens should *not* be submitted for routine diagnostic tests. Reference specimens may be submitted only by the State health department laboratory. They must conform with postal regulations, as well as with NCDC instructions for labelling and transmitting specimens, and for packaging specimens for air mail shipments. Specimens arriving in damaged condition will be destroyed without examination.

The appropriate receiving laboratory should be checked on the mailing label to insure prompt delivery of specimens. Specimens should be mailed so as *not* to arrive in the laboratory on a Saturday, Sunday, or holiday. Except as indicated on the list below, specimens sent to NCDC laboratories should be accompanied by a diagnostic report form available from the NCDC.

Laboratory support to epidemiological investigations, survey studies, or special research projects should be arranged by prior consultation with the Chief of the Laboratory Program.

REFERENCE SERVICES AVAILABLE	RECEIVING LABORATORY
Bacteriology	
Identification, toxigenicity testing, and typing of *C. diphtheriae* Slide agglutination and microscopic tests for leptospirosis. Submit form PHS 3.203E with these specimens. Febrile agglutination tests Serotyping of *Listeria* Sera for the diagnosis of infections with *S. typhi* and other *Salmonella* types accepted only under exceptional circumstances. Sera for agglutination tests for diagnosis of bacillary dysentery are not accepted because they are of no value for this purpose.	Bacterial Serology Unit
Grouping of meningococci. Drug sensitivity testing. Identification of miscellaneous bacteria other than the *Enterobacteriaceae*, streptococci, *C. diphtheriae,* and mycobacteria. (Miscellaneous bacteria cannot be accepted for identification unless they are accompanied by a complete history.) Identification of anaerobic bacteria and their toxins. Bacteriophage typing of staphylococci. (Only epidemiologically related cultures will be accepted.) Focal point for processing of bacteriology intrastate laboratory evaluation specimens.	Bacterial Reference Unit
Serological grouping of beta hemolytic streptococci and typing of Group A and Group B streptococci. Biochemical differentiation of other streptococci and of pneumococci. Serotyping of pneumococci. Micro-antistreptolysin 0 determinations.	Streptococcus Unit
Identification of *Salmonella, Shigella,* and Arizona types. Bacteriophage typing of *S. typhi, S. paratyphi* B, and *S. typhimurium.* Typing of *Escherichia* cultures from outbreaks of diarrhea in infants. Typing of *Klebsiella* cultures in particular instances. Identification of other enteric bacteria as warranted by clinical and epidemiological considerations.	Enteric Bacteriology Unit
Identification of acid-fast bacilli. Determination of drug susceptibility of acid-fast bacilli.	Tuberculosis Unit
Serotyping of leptospira cultures isolated from human, animal, or environmental sources.	Veterinary Public Health Laboratory Unit (Epidemiology Program)

Mycology	
Examination of cultures for pathogenic fungi (Dermatophytes, subcutaneous and systemic pathogens). Examination of stained and unstained histological slides for presence of pathogenic fungi. Examination of clinical materials from cutaneous mycotic diseases. (Hair, skin scrapings, nail clippings, etc.) Examination of cultures and clinical materials of veterinary origin. Serologic tests for actinomycosis, blastomycosis, coccidioidomycosis, cryptococcosis, and histoplasmosis.	Mycology Unit
Parasitology	
Examination of feces, urine, sputum, and other body fluids and aspirates for evidence of parasitic infection. (*Entamoeba histolytica* and other intestinal protozoa, intestinal helminths, schistosomes, *Paragonimus westermani, Echinococcus granulosus,* etc.) Examination of biopsy or autopsy material for *Trichinella spiralis, Echinococcus granulosus,* etc. Examination of histological preparations for presence of parasites. Identification of isolated worms. Examination of thick or thin blood films, impression smears, tissue sections, etc., for blood parasites (malarial parasites, hemoflagellates, filarial worms, etc.) Isolation of hemoflagellates by cultivation and animal inoculation from unpreserved whole blood, spinal fluid, tissue, aspirates, etc. Serologic tests for toxoplasmosis, trichinosis, echinococcosis, extra-intestinal amebiasis, visceral larva migrans, cysticercosis, filariasis, schistosomiasis, Chagas disease, and kala-azar. *By special arrangement:* examination of impression smears, spinal fluid, tissue sections, etc., for *Toxoplasma;* attempted isolation of organisms from whole blood, spinal fluid, tissues, etc.; serology on adult eye lesion cases.	Parasitology Unit
VD Laboratory Procedures	
Treponema Pallidum Immobilization (TPI) test on serum. Identification of cultures of *Neisseria gonorrhoeae* Under certain special conditions approved by the Venereal Disease Research Laboratory, cultures of gonococci may be accepted for testing to determine susceptibility to penicillin.	Venereal Disease Research Laboratory (Venereal Disease Program) ·
Virology and Rickettsiology	
Reference studies for the identification of infectious agents in tissue culture fluids, animal tissues, or original clinical materials (including acute and convalescent serum specimens for serology) submitted for confirmation or extension of initial findings and examination of specimens of animal origin for studies involving viral agents transmissable to man. (Form PHS 3.332 should accompany these specimens.)	Virus Reference Unit

APPENDIX V

DEPARTMENT OF
HEALTH, EDUCATION, AND WELFARE
Public Health Service   Communicable Disease Center
Laboratory Branch   Virology Section
Atlanta, Georgia 30333

REQUEST FOR VIRAL AND RICKETTSIAL REFERENCE SERVICE

For CDC Use Only

Patient's Name _____

Address _____

City _____

County _____ State _____

Age _____ Race _____ Sex _____

Occupation _____

Date of Onset _____

Clinical Diagnosis _____

_____

_____

Physician _____

Address _____

_____

CDC LAB. NO. _____

Date Received _____

Local File No. _____

State Approval _____

Referral _____

Other Source _____

_____

_____

LABORATORY EXAMINATION REQUESTED:

Suspected Virus Group _____

VIRUS: Isolation _____ Identification _____

SEROLOGY _____ OTHER _____

REASON FOR REQUEST:

Epidemic Investigation _____

Surveillance Activities _____

Epidemiologic Survey _____

Other _____

OTHER CLINICAL DATA:

DATES OF PERTINENT IMMUNIZATIONS

Adenovirus _____

Influenza _____

Polio - Salk: No. _____ _____

         Oral: Type_____ _____

_____ _____

_____ _____

Smallpox _____   Other

Spotted Fever _____ _____

Typhus _____ _____

Yellow Fever _____ _____

Rabies _____ _____

Measles _____ _____

EPIDEMIOLOGICAL DATA

Recent Travel (Location) _____

Family Contacts _____

Community Contacts _____

Animal Contacts _____

Arthropod Contacts:	Mosquitos	Ticks	Sandflies	Other Arthropods
Bite	_____	____	_____	_____
Exposure only	_____	____	_____	_____

SIGNS AND SYMPTOMS

Fever: Height _____, Duration _____ .

Rash : (Type) _____ .

Mucous Membrane lesions _____

Respiratory: Rhinitis _____   Pharyngitis _____

                Pneumonic involvement _____

Cardiovascular: Myocarditis _____   Pericarditis _____

Gastrointestinal: Diarrhea _____   Constipation _____

                 Abdominal pain _____   Vomiting _____

(Continued on Reverse Side)

Central Nervous System: Headache _____ Delerium _____ Seizures _____ Lethargy _____
Paralysis: Flaccid _____ Spastic _____
Muscle Weakness _____ Bulbar Involvement _____
Meningismus _____ Nuchal Rigidity _____
Other _____

Others: Jaundice _____ Myalgia _____ Pleurodynia _____
Myositis _____ Conjunctivitis _____
Hemorrhagic Phenomena _____ Hepatomegaly _____

Associated Illness: _____

Fatal _____

TREATMENT

Antibiotics _____
Other _____

SPECIMENS SUBMITTED FOR EXAMINATION:

Origin: Human _____ Animal _____ Other _____

Specimen	Date of Collection	Specimen	Local File No.	Date of Collection		
Stool	_____	Serum: Acute	_____	_____		
	_____	Convalescent	_____	_____		
Throat Washing	_____		_____	_____		
	_____		_____	_____		
Spinal Fluid	_____					
	_____	Virus Isolate:		Source		Passage
Others _____	_____	Tissue Culture _____	_____	_____		_____
_____	_____	Mouse _____	_____	_____		_____
		Egg _____	_____	_____		_____
		Other _____	_____	_____		_____

STORAGE OF SPECIMENS: Duration _____ Method _____

LOCAL LABORATORY RESULTS:

Clinical diagnostic: WBC _____ Differential _____
_____

CSF: Cell Count _____ % Lymphocytes _____
Others _____
Liver Function Studies _____
Bacteriological Studies: _____
Pathological Examinations: _____
Virus Isolation: Specimen _____ Method _____
Results _____
_____

Serology: Method used: CF _____ Neut. _____ HI _____

Test Antigen										
Acute										
Convalescent										

Other Pertinent Information: _____
_____
_____

44

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