

Guidelines on Standard Operating Procedures for Laboratory Diagnosis of HIV-Opportunistic Infections

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World Health Organization
Regional Office for South-East Asia
New Delhi
June 2001

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Foreword

Standard Operating Procedures (SOPs) are an essential part of good laboratory practices. These provide a stable pattern of function by the laboratory staff and ensure consistency of quality in laboratory results. SOPs are also a prerequisite for accreditation of laboratories. WHO has been providing technical support in various laboratory technologies by providing guidelines for the development of SOPs. Guidelines on Standard Operating Procedures for Laboratory Diagnosis of HIV-Opportunistic Infections is another step in this direction.

HIV/AIDS is perhaps the most important challenge being faced by the human race. In spite of considerable efforts and inputs, specific antiviral therapy that is accessible and affordable to the growing number of HIV/AIDS cases in developing countries could not be provided so far. These patients with a weakened immune system contract opportunistic infections. It is estimated that the problem will confront Member Countries of the South-East Asia Region with greater vengeance in the days to come. An early diagnosis of these infections is of vital importance for better management and initiation of preventive measures.

Opportunistic infections differ from conventional communicable diseases and have assumed importance only after the advent of HIV/AIDS. Understandably, the expertise for the diagnosis of these infections is sub-optimal in the developing countries. Guidelines on Standard Operating Procedures for Laboratory Diagnosis of HIV-Opportunistic Infections is aimed at providing technical support to laboratories that are involved in diagnosing opportunistic infections. On the basis of these guidelines, the laboratories need to develop their SOPs and train their staff in their effective use. Periodic review and updating of these SOPs in the light of new knowledge and technology are also suggested.

I am sure this publication will meet its intended objectives and help mankind in its fight against the menace of HIV/AIDS.



Dr Uton Muchtar Rafei
Regional Director

Preface

Opportunistic infections (OIs) are caused either by organisms of low or no virulence which are non-pathogenic in individuals with an intact immune system, or by known pathogens who present in a different way than usual in immunodeficient individuals in the form of increased virulence, recurrence, multi-drug resistance or atypical presentation.

The spectrum of opportunistic infections has been found to vary from continent to continent and region to region. With the unprecedented increase in the number of AIDS cases, OIs are also increasing. The early diagnosis of these infections is vital for better management and preventive measures. There are misconceptions about the diagnosis of these infections. A consultative group meeting was organized at the WHO Collaborating Centre for Training and Research on HIV/AIDS, Clinical Management and Counselling, in the Department of Communicable Disease Control, Ministry of Public Health, Thailand, from 27-30 September 1999 to finalize Standard Operating Procedures (SOPs) for commonly encountered OIs in the South-East Asia Region.

Guidelines for preparation of SOPs are presented in six chapters dealing with the diagnosis of HIV infection and bacterial, viral, parasitic and fungal OIs commonly associated with AIDS. An exclusive chapter has been included for the laboratory diagnosis of tuberculosis and Mycobacterium avium complex disease in HIV-infected patients, since tuberculosis has been found to be the most commonly encountered OI in AIDS patients in the South-East Asia Region.

SOPs for these diseases focus on specimen collection, storage and transportation, specimen processing, examination, identification, recording and reporting, interpretation of results, quality assurance procedures and safety precautions. Tests and methods, which are either in use or can be easily adopted in most of the laboratories in the Member Countries have been included. References have been provided for further reading on the subject.

It is hoped that this publication will benefit immensely laboratories in the Member Countries in establishing facilities for diagnosis of OIs. There is a continual need to update and periodically review the SOPs in each laboratory based on its local requirements.

We welcome any feedback, comments and suggestions on this publication for incorporation in the next edition.

Acknowledgements

The drafts on Guidelines for Preparation of Standard Operating Procedures were finalized during a Consultative Meeting on Standard Operating Procedures for Diagnosis of HIV-Opportunistic Infections organized by Dr Pikul Moolasart at the WHO Collaborating Centre for Training and Research on HIV/AIDS, Bamrasnaradura Hospital, Nonthaburi, Thailand from 27-30 September 1999.

The valuable contributions of all the experts listed in the Annex are gratefully acknowledged.

1. Standard Operating Procedures for Diagnosis of HIV Infection

Introduction

This SOP is confined to the standard techniques of anti-HIV testing as per WHO testing strategies.

Clinical manifestations

Human immunodeficiency virus (HIV) infection can lead to a variety of diseases. In primary infection, some adolescents or individuals may experience the acute retroviral syndrome at or near the time of seroconversion, then recover and remain asymptomatic for years before progression to the full-blown disease of Acquired Immunodeficiency Syndrome (AIDS).

Laboratory diagnosis

The standard test for diagnosis of HIV infection is the serology for antibody detection. HIV antibody assays are now commercially available in various formats. The adverse economic, social and psychological cost of false positive and false negative assays for HIV infection have pushed investigators and manufacturers to develop diagnostic anti-HIV test kits with high sensitivity and specificity.

There are two types of HIV: HIV-1 and HIV-2. HIV-1 is divided into distinct genetic subtypes. The anti-HIV assay should include the antigen for detection of anti-HIV-1, anti-HIV-2 and HIV-1 subtype O antibodies.

ELISA is the most widely used anti-HIV antibody test. The principles of ELISA are classified as indirect, competitive and sandwich test. The competitive principle is not popular because of the low sensitivity. The antigens used are prepared from viral lysate or recombinant proteins and/or synthetic peptides. The ELISA techniques require an ELISA Reader and are suitable for use in the laboratories where more than 30 samples are tested at each time. Gelatin particle agglutination (GPA) is a simple test and can be used as an alternative or supplemental test to ELISA.

Rapid tests are visual tests that do not require the ELISA Reader. These tests are available in smaller test packs and therefore, are suitable for a laboratory having smaller sample numbers. They are technically simpler to perform although most of them have sensitivity and specificity comparable to ELISA. The commonly employed rapid anti-HIV antibody tests are based on the principles of dot immunoassay, or particle agglutination (e.g. gelatin or latex).

Western blot which is the standard confirmatory test should only be used to resolve indeterminate results and diagnosis of HIV-2.

The specimen of choice for anti-HIV testing is serum or plasma. However, assays for detection of anti-HIV in whole blood, saliva/oral fluid, urine and dried blood spot have also been developed. These specimens have been utilized for surveillance purposes.

In primary infection, the virus in the blood could be demonstrated by nucleic acid-based test (PCR for proviral DNA and RT-PCR for viral RNA), p24 antigen testing or culture. In most cases, the period of viremia is approximately less than one week prior to the appearance of antibody. Antibodies to HIV are detectable within four to six weeks of infection by commonly employed tests and in virtually all infected individuals within six months and it persists for life. The level of viremia is a predictive marker of HIV disease progression.

Diagnosis of HIV infection in babies born to HIV-infected mothers cannot be established by conventional antibody tests. This is because the presence of anti-HIV antibody in the newborn may not necessarily indicate primary infection, but may also be due to passive transmission of anti-HIV antibody from mother to the uninfected child that may persist even up to 18 months. Detection of viral RNA, or viral DNA, viral culture, or detection of the IgA class of antibodies to HIV (IgA does not pass placenta) are more appropriate methods for diagnosis of HIV infection at the early stage (*i.e.* less than 18 months).

Safety considerations

Laboratory safety must be strictly followed according to good laboratory practice (GLP) guidelines and universal precautions. All laboratory accidents should be listed and documented. Laboratory personnel should not be allowed to work alone and must be familiar with, and use at all times aseptic or sterile techniques.

Specimen collection	<ul style="list-style-type: none">Specimens should be collected with universal precautions and put in sterile leak proof unbreakable containers.
Specimen transport	<ul style="list-style-type: none">Specimens should be sent to the laboratory as soon as possible.
Specimen processing	<ul style="list-style-type: none">Specimens should be separated in a closed system centrifuge.

Specimen collection

Optimal time of specimen collection	<ul style="list-style-type: none">Blood specimens could be collected at any convenient time.
Correct specimen type and method of collection	<ul style="list-style-type: none">Whole blood or anticoagulated blood
Adequate quantity and appropriate number of specimens	<ul style="list-style-type: none">Approximately 3-5 ml. An additional blood sample is required subsequently if the first sample gives a positive result.

Specimen transport and storage

Time between specimen collection and processing	<ul style="list-style-type: none"> Specimens should be transported and processed as soon as possible or within 24 - 48 hours.
Special considerations to minimize Deterioration	<ul style="list-style-type: none"> If serum/plasma has been separated, it can be stored in a refrigerator for a week or in a freezer at -20°C or lower temperature for a longer period.

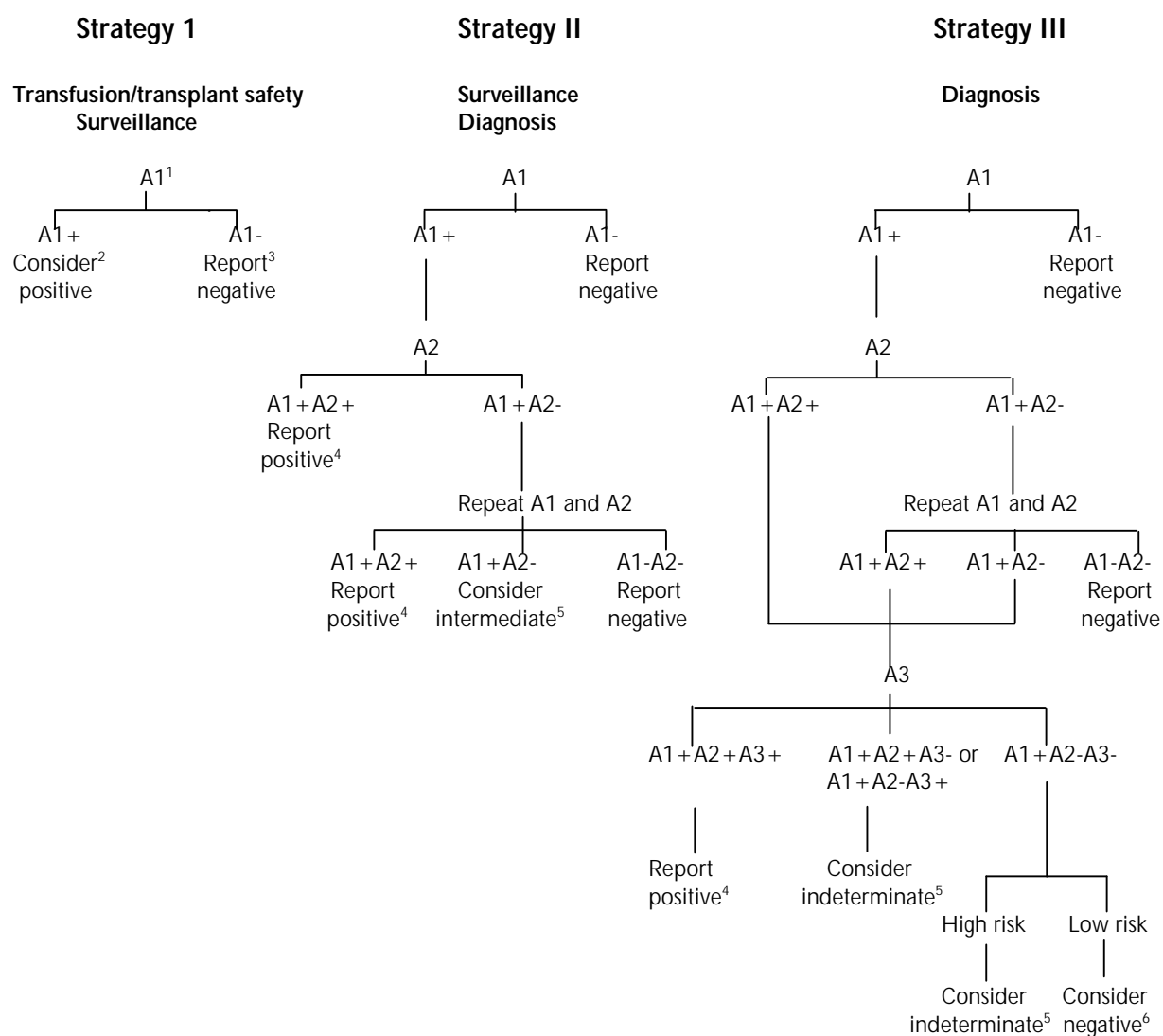
Specimen processing

Test selection	<ul style="list-style-type: none"> High quality reagents (ELISA or other simple/rapid assays) should be selected as appropriate.
Appearance	<ul style="list-style-type: none"> The appearance of the sample should be checked for evidence of hemolysis, jaundice or lipemia.
Strategy for anti-HIV testing	<ul style="list-style-type: none"> The strategy of HIV testing is based on the objective of testing the prevalence of HIV in the population. For details see Table 1 and Figure 1.
Confirmatory Test	<ul style="list-style-type: none"> In populations where the prevalence of HIV infection is greater than 10%, the initial reactive specimen will be tested by a different screening test (e.g. ELISA or simple/rapid) based on different antigen preparation (e.g. viral lysate and recombinant antigen) or different test principles (e.g. indirect ELISA and sandwich ELISA). Any serum that is reactive on the first test but non-reactive on the second test, should be retested with the two assays. Concordant results after repeat testing will indicate a positive or negative result. If the results of the two assays remain discordant the serum is considered indeterminate. In populations in which the prevalence of HIV infection is less than 10%, after the results of the two assays are concordantly positive, the third test is employed. For the sera found to be repeatedly discordant <i>i.e.</i> positive by first and negative by second test the third test is also employed. The three tests must be based on different antigen preparations or test principles. Serum that is reactive by all the three tests is considered positive. Serum that remains discordant in the second test or is reactive in the first and second test but non reactive in the third test is considered indeterminate.

Table 1: UNAIDS and WHO recommendations for HIV testing strategies according to test objective and prevalence of infection in the sample population.

Objective of testing	Prevalence of infection	Testing strategy
Transfusion/Transplant safety	All prevalence	I
Surveillance	> 10%	I
	≤ 10%	II
Diagnosis	> 30%	I
	≤ 30%	II
	> 10%	II
	≤ 10%	III

Figure 1: Schematic representation of the UNAIDS and WHO HIV testing strategies



¹Assay A1, A2 and A3 represent three different assays

² Such a result is not adequate for diagnostic purposes: use strategy II or III. Whatever the diagnosis, donations which were earlier reactive should not be used for transfusion or transplant

³Report: result may be reported

⁴For newly diagnosed individuals a positive result should be confirmed on a second specimen

⁵Testing should be repeated on a second specimen taken after 14 days

⁶Result is considered negative in the absence of any risk of HIV infection

The follow-up sample from patients with indeterminate result should be collected after two weeks. If the second sample also shows indeterminate result, it should be tested by a confirmatory assay (e.g. Western Blot). However, if the confirmatory test fails to resolve the serodiagnosis, follow up testing should be undertaken at four weeks, three months, six months and 12 month-intervals. After 12 months, such indeterminate results should be considered as negative.

Reporting procedure

The results are kept confidential.

Report	<ul style="list-style-type: none">• Negative - If the initial/screening test shows non-reactive result.• Positive - If the sample shows reactive results concordantly by three screening tests. The subsequent sample is requested to retest before the result is reported to the patient.• Indeterminate - If the sample shows discordant results by the three screening tests, the follow up samples are required to retest at two weeks and at three, six and 12 months before the result is reported to the individual. If the results remain indeterminate after one year, the person is considered to be HIV antibody negative.
Special considerations	<ul style="list-style-type: none">• Pre test-, post test-counselling service should be provided and the result should be kept confidential.

Reference

WHO (March 1997) Revised Recommendation for the Selection and Use of HIV Antibody Tests. Weekly Epidemiological Record, No. 12.

2. Standard Operating Procedures for Laboratory Diagnosis of Viral Opportunistic Infections in HIV-Infected Patients

Introduction

This SOP is confined to simple techniques for diagnosis of herpes simplex virus (HSV), varicella-zoster virus (VZV) and cytomegalovirus (CMV) which are the common opportunistic viral infections in HIV-infected persons.

Clinical manifestations

Herpes simplex virus type 1 (HSV 1) and type 2 (HSV 2) infections cause primary and recurrent oral, genital and rectal ulceration and occasionally disseminated visceral and central nervous system disease.

VZV causes varicella in primary infection, and herpes zoster when reinfected later in lifetime. In HIV-infected persons, reactivation of VZV cause prolonged and severe manifestation of herpes zoster and occasionally becomes disseminated.

CMV infection is responsible for a greater proportion of opportunistic infections in HIV-infected persons with advanced immune deficiency. Retinitis is the most frequent clinical manifestation of CMV infection in patients with AIDS. Gastrointestinal disease, encephalitis, polyradiculitis, and pneumonia may also occur.

Laboratory diagnosis

Viral diagnosis is mostly based on clinical symptoms and signs. The most common technique is simple microscopic examination of scraped cells for diagnosis of HSV skin, oral, genital and anal lesions and VZV lesions (Tzanck test). Demonstration of multinucleated giant cells indicates infection by either HSV or VZV. The two viruses could be differentiated by immunological techniques *i.e.* immuno-fluorescence, immunoperoxidase and ELISA.

Detection of CMV can be done by conventional culture, PCR or the semiquantitative antigenemia assay. Demonstration of the virus in affected tissue confirms the diagnosis of CMV disease (*e.g.* bronchoalveolar lavage specimen, or biopsy and necropsy specimens). Detection of a high level of the virus in blood by a quantitative assay is predictive of CMV disease. Viral shedding in urine indicates reactivation and may be associated with CMV disease.

Serological diagnosis is useful in primary herpes virus infection, but not in reactivation and immunodeficiency states.

Viral culture and PCR are the techniques available in reference laboratories. Specimen collection, transportation and storage are described in this SOP.

The other viruses associated with HIV-infected persons are: Epstein Barr virus (EBV); Kaposi's sarcoma-associated herpes virus (HHV 8); Other viruses, e.g. human papillomaviruses (HPV), molluscum contagiosum virus, hepatitis B virus (HBV), and hepatitis C virus (HCV). Investigation of these viruses is carried out in the reference and research laboratories.

Safety considerations

Specimen collection	<ul style="list-style-type: none"> • Universal precautions and avoidance of contact with lesion or tissue with ungloved hands.
Specimen transport and storage	<ul style="list-style-type: none"> • Sterile leak-proof container in a sealed plastic bag.
Specimen processing	<ul style="list-style-type: none"> • Biosafety level II or III with good laboratory practice.

The above guidelines should be supplemented with recommendations from the local health hazard and risk assessment committee.

Specimen collection

Optimal time of specimen collection	<ul style="list-style-type: none"> • At the time close to onset or as early as possible. • Before the start of antiherpetic drugs
Correct specimen type and method of collection	<ul style="list-style-type: none"> • Skin and mucosa lesions: Collect from newly formed vesicles. Using sterile No. 27 needle and tuberculin syringe, aspirate the vesicular fluid for culture. Open the lesion and scrape the cells at the rim of the base of the lesion. Smear on dry clean glass slides (2-3 slides) • Swab from the open lesion, immerse in viral transport media. • Cerebrospinal fluid: Collect 1-2 ml in sterile container. • Biopsy or necropsy tissues: Try to avoid contamination as much as possible. Collect in sterile container. • Blood: Collect venous blood 3-5 ml in EDTA tube. • Bronchoalveolar lavage: Collect 10 ml in sterile container.

Specimen transport and storage

Time between specimen collection and processing	<ul style="list-style-type: none"> • All specimens should be fresh and sent to the laboratory as soon as possible. • Slides from skin or mucosal lesion smears should be air dried and sent in sealed plastic bags. • Vesicular fluid and tissue specimens should be kept in sterile containers and sealed plastic bags, kept refrigerated and transported in an ice-box. • Lesion swabs should be put in sterile tubes with appropriate amounts of viral transport media, kept in refrigerator and transported in an ice-box. • Blood specimen should be transported at room temperature.
Special considerations to minimize deterioration	<ul style="list-style-type: none"> • Specimens should be processed as soon as possible especially for viral isolation. • For CMV antigenemia assay, specimens should be processed within six hours.

Specimen processing

Proper documentation upon receipt of specimen	<ul style="list-style-type: none"> • Laboratory request form should indicate the specific test for HSV, VZV and CMV. • Record in laboratory file and give laboratory number.
Processing	<ul style="list-style-type: none"> • Smears are fixed in absolute methanol and stained with Giemsa stain. • Smears are fixed in acetone and sent to a reference laboratory for staining with specific anti-HSV or anti-VZV as requested. • Specimens requested for PCR, viral culture and CMV antigenemia assay are referred to a reference laboratory.

Reporting procedure

Microscopy	Giemsa stain : Positive or Negative for multinucleated giant cell. Inadequate specimen if no epithelial cell is found.
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Giemsa staining

To prepare Giemsa stock solution, dissolve 0.5 gm of Giemsa powder in 33 ml of glycerine by placing the mixture in a water bath (55°-60°C) for 90 minutes. When the powder is dissolved, add 33 ml of absolute methanol. The stock solution is stored at room temperature.

The scraping prepared onto clean glass slide is fixed in absolute methanol for ten minutes. The slides are then stained with Giemsa stain (freshly prepared by diluting the stock solution 1:20 in water) for two hours. The stained slides are decolourized in 95% ethanol for a few seconds and rinsed in tap water.

Reference

WHO(2000) Malaria. In Guidelines on Standard Operating Procedures for Microbiology (SEA/HLM/324) WHO Regional Office for South-East Asia, New Delhi, p. 119.

3. Standard Operating Procedures for Laboratory Diagnosis of Tuberculosis and *M. avium* Complex Disease in HIV-Positive Patients

Introduction

HIV has a serious effect on the tuberculosis control programme in many parts of the world. Tuberculosis is considered to be one of the most common HIV-related opportunistic infections. The frequency with which HIV and *M. tuberculosis* infections occur together is determined by the epidemiology of each disease in a given population. Although several population-based studies determining the incidence of tuberculosis attributed to HIV infection have been documented elsewhere, such reliable information is not readily available for all SEAR countries. However, the few studies reported from India, Thailand and elsewhere among SEAR countries reveal that tuberculosis is the most common infection in the HIV-infected group.

Before the AIDS pandemic, Non-Tuberculous Mycobacteria (NTM) rarely caused serious illness, even in the immunocompromised individuals. The prolonged immunosuppression of the cell mediated immune system caused by the HIV provides the opportunity for these relatively avirulent organisms to cause disease. *M. avium* complex (MAC) was recognised in the early AIDS pandemic as a cause of serious disseminated infection and is now the most common cause of systemic bacterial infection in AIDS, affecting 15% to 40% of patients in the United States, although such a scenario has not been found so far in the SEAR countries.

Tuberculosis in HIV-infected persons may occur with different manifestations. All these forms of tuberculosis, except when cavitation occurs in pulmonary tuberculosis, are paucibacillary in nature. Depending upon the form of disease manifestation, several specimens such as sputum and/or gastric lavage, bronchoalveolar lavage (BAL), lymph nodes and other biopsy specimens, pus, ascitic fluid, pleural and cerebrospinal fluid should be examined. If delay is anticipated, biopsy specimen may be collected in suitable transport medium for sending them to the laboratory.

Laboratory diagnosis

There are two ways to address the diagnosis of tuberculosis. The direct approach is concerned with the detection of tubercle bacilli by smear and culture or detection of its products such as detection of tuberculostearic acid, detection and identification of mycobacterial antigens by the use of polyclonal or monoclonal antibodies, analysis of mycolic acids by chromatography, and the detection of DNA or RNA of mycobacterial origin by hybridization with DNA probes with or without amplification of nucleic acids.

The indirect approach relates to the measurement of host immune response against the mycobacteria. This includes humoral immunity via the detection of antibodies against the bacteria and cellular response via skin tests.

None of the above parameters except smear microscopy, culture on LJ medium, minimum set of identification to differentiate *M.tuberculosis* from NTM and drug susceptibility testing by the conventional method, are practicable in most of the SEAR countries. And also since contribution of pulmonary tuberculosis accounts for more than 90% of total tuberculosis manifestation in HIV patients, this SOP is confined to the processing of sputum sample for *M.tuberculosis* and its identification and drug susceptibility testing methods. In addition, brief general identification of NTM and details pertaining to processing of infectious material for MAC disease are also provided.

Collection of sputum

For diagnosis of tuberculosis, three specimens of sputum are to be examined (spot-morning - spot) over a period of two days. Specimens are to be collected in sterile universal containers, and should have a fixed label for noting patient's information on the side of the container.

Specimen collection	<ul style="list-style-type: none"> Aerosol free container (Sputum cup with a lid or Mc Cartney bottle)
Adequate quantity and Appropriate number of specimens	<ul style="list-style-type: none"> Ideally, a minimum volume of 5 ml Three consecutive sputum specimens should be collected: spot – morning – spot sputum.
Specimen transport and storage	<ul style="list-style-type: none"> Sterile leak proof container in a sealed plastic bag. To be transported within three days for culture. If delay is anticipated, add equal volume of 1% CPC.
Specimen staining	<ul style="list-style-type: none"> Ziehl-Neelsen staining Note: heat-fixing may not kill all <i>Mycobacterium</i> species. Slides should be handled carefully
Culture and investigation	<ul style="list-style-type: none"> Specimen processing Sputum: Modified Petroff's method for digestion and decontamination. Other samples: Direct inoculation for aseptic specimens. Mild acid or other standard pretreatment procedure. Inoculation on Lowenstein-Jensen media or Lowenstein-Jensen with sodium pyruvate used for INH Resistant strain and <i>M. bovis</i>. Other specimens: using Lowenstein-Jensen media and one bottle of liquid medium (Middlebrook 7H9 or Kirchner's medium). Incubation at 37°C for <i>M. tuberculosis</i> and 25°C, 37°C and 45°C for NTM. Examination for eight consecutive weeks or until it becomes positive or contaminated.
Culture identification	<ul style="list-style-type: none"> <i>M. tuberculosis</i> Growth rate slow grower (> 7 days) Pigmentation buff colour Niacin test + Nitrate reduction test + Catalase at 68°C - Growth in PNB no growth Note: Identification for NTM is contained in separate SOPs.

Drug susceptibility testing (DST)	<ul style="list-style-type: none"> DST is undertaken against streptomycin, isoniazid, rifampicin and ethambutol on L-J medium. Method: Using a standard proportion method. 															
Definition of resistance	<ul style="list-style-type: none"> The number of organisms resistant to each drug concentration as a percentage of the number of organisms growing on the drug-free slope. <table border="1"> <thead> <tr> <th><u>Drug</u></th> <th><u>Concentration</u></th> <th><u>Proportion</u></th> </tr> </thead> <tbody> <tr> <td>Streptomycin</td> <td>4 mg/l</td> <td>1% or more</td> </tr> <tr> <td>Isoniazid</td> <td>0.2 mg/l</td> <td>1% or more</td> </tr> <tr> <td>Rifampicin</td> <td>40 mg/l</td> <td>1% or more</td> </tr> <tr> <td>Ethambutol</td> <td>2 mg/l</td> <td>1% or more</td> </tr> </tbody> </table>	<u>Drug</u>	<u>Concentration</u>	<u>Proportion</u>	Streptomycin	4 mg/l	1% or more	Isoniazid	0.2 mg/l	1% or more	Rifampicin	40 mg/l	1% or more	Ethambutol	2 mg/l	1% or more
<u>Drug</u>	<u>Concentration</u>	<u>Proportion</u>														
Streptomycin	4 mg/l	1% or more														
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Rifampicin	40 mg/l	1% or more														
Ethambutol	2 mg/l	1% or more														
Recording and Reporting	<ul style="list-style-type: none"> Direct smear <ul style="list-style-type: none"> +++ (> 10 AFB/oil field) ++ (1 – 10 AFB/oil field) + (10 – 99 AFB/100 oil fields) scanty (1 – 9 AFB/100 oil fields) negative (0 AFB/100 oil fields) Culture <ul style="list-style-type: none"> +++ (confluent growth) ++ (innumerable discrete colonies) + (20 - 100 colonies) actual count (< 20 colonies) 															
Quality assurance	<ul style="list-style-type: none"> Smear microscopy Re-reading both positive and negative slides with blind results. Culture Using the suspension of H37Rv to check the L-J medium. Note: <i>The contamination rate should not more than 5%</i> Drug susceptibility testing <ul style="list-style-type: none"> - Internal quality control - External quality assessment programme with Supranational reference laboratory 															

Diagnosis of AIDS and MAC

Specimen type and method of collection	<ul style="list-style-type: none"> Blood, bone marrow, biopsy specimen and others. All specimens should be fresh and taken before antimicrobial treatment is started.
Specimen processing	<ul style="list-style-type: none"> Hemoculture, Culture of tissue materials
Culture media	<ul style="list-style-type: none"> Lowenstein-Jensen media, Middlebrook 7H12, Bactec 13A or MB/BacT
Identification	<ul style="list-style-type: none"> Slow grower Growth at 45°C Non-pigmented Catalase + Pyrazinamidase + Other molecular methods

Sputum smear Microscopy

Microscopy of sputum is of great value in the detection of open or infectious cases of tuberculosis. The establishment of a good sputum microscopy service is of prime importance in developing countries for detection and treatment of the open cases.

Smears are stained by the Ziehl-Neelsen (ZN) method, or by one of its various modifications. Grading of the positive smears gives a broad indication of the severity of disease and the response to therapy (during treatment of the patient).

The patient is given a container on his first attendance. He should be instructed, with demonstration by actual actions, to

- inhale deeply 2-3 times
- cough out deep from the chest
- open the container and spit the sputum into the bottle
- avoid saliva or nasal secretions
- close the container.

A good sputum sample should be thick, purulent and there should be sufficient quantity (at least 5ml).

The patient should be instructed to collect an early morning specimen in a similar manner and bring it the next morning. On the second attendance, a third specimen (spot) is collected.

The details of the patient's, name, address, age/sex and bottle no. should be recorded in a form/ card and sent to the laboratory.

On receipt in the laboratory, the specimens are given serial laboratory numbers.

Collection of other specimens⁽¹⁾

Prepare smears from other specimens as for sputum, but do not expect all liquid specimens to produce a cloudy smear.

All liquid specimens, except blood, should be decontaminated and concentrated by centrifugation before smears and cultures are made.

Tissue specimens are frequently cut into thin sections on a microtome, are attached to a glass slide, and acid-fast stained. This procedure requires special equipment and training and will not be discussed in this manual. Tissue specimens may also be ground to a fine pulp for culture and microscopy.

Other specimens can be done for decontamination as sputum specimens as well.

Smear examination

The laboratory number is written, with a diamond marker, on one edge of a new, clean, grease-free and unscratched slides. Using a broomstick or a wire loop (5 mm diameter), a

purulent portion of the sputum is placed on the slide and spread evenly to give a smear of approximately 3 x 2 cm. The smear is allowed to air-dry and fixed by pressing over a flame 3-5 times for 3-4 seconds each time.

Staining procedure

- (1) Place the fixed slides on a staining rack with the smeared side facing up
- (2) Pour 1% carbol fuchsin to cover the entire slide
- (3) Heat the slides from below till vapour rises
- (4) Allow the slide to stand for at least five minutes
- (5) Rinse gently with tap water to remove excess stain
- (6) Decolourize using 25% sulphuric acid for 2-4 minutes
- (7) Rinse gently again with water
- (8) Counterstain with 0.1% methylene blue for 30 seconds
- (9) Rinse gently again and allow the slide to air dry

Smear reading

Place a drop of cedarwood oil or liquid paraffin without touching the smear and examine under an oil-immersion objective for at least 10 minutes. Count the number of AFB and grade as 3+, 2+, 1+, scanty or negative as follows:

Grading of AFB smears

Examination	Result	Grading	No. of fields to be examined
More than 10 AFB per one oil immersion field	Positive	3+	20
1-10 AFB per one oil immersion field	Positive	2+	50
10-99 AFB per 100 oil immersion fields	Positive	1+	100
1-9 AFB per 100 oil immersion fields	Scanty	Record exact number seen	100
No AFB per 100 oil immersion fields	Negative	-	100

Sputum culture (Modified Petroff's Method)*

- (1) Discard sputum in excess of 5 ml into the disinfectant bath (5% Phenol).
- (2) To each volume of remaining sputum add two volumes of 4% sodium hydroxide, taking care to avoid contact between the specimen bottle rim and the sodium hydroxide flask.

* The total period of contact with the alkali is 35 minutes. It has been shown that this period of contact with 4% NaOH may not be lethal to *tubercle bacilli*. However, in the event of longer duration of contact with the alkali, neutralize the NaOH by adding hydrochloric acid before centrifugation. ^(3,4)

- (3) Screw the caps firmly on, ensuring at the same time that the bottle screw tops are not broken or chipped.
- (4) Shake the bottles by hand for one minute.
- (5) Place in a rack on the shaking machine and leave to shake gently for 20 minutes.#
- (6) Remove the specimens from the shaker.
- (7) Centrifuge for 15 minutes* at 4000 rpm (approx. 3000g).
- (8) Carefully pour off the supernatant into a disinfectant bath.
- (9) Fill the bottles with approximately 20 ml of sterile distilled water, shake by hand to mix the deposit, re-centrifuge for 15 minutes and pour off the supernatant as before.
- (10) Finally inoculate each sediment with a 5 mm diameter loop onto two previously numbered Lowenstein-Jensen slopes and one slope of L-J enriched with sodium pyruvate.
- (11) Place the inoculated media in the 37°C incubator.

Culture reading

Cultures which have been incubated are examined on a fixed day of the week for 8 consecutive weeks or until they become positive or contaminated.

Record the final results at the time of reading: negative, contaminated and if positive, the degree of positivity as follows:

+++	confluent growth
++	innumerable discrete colonies
+	20 - 100 colonies
Actual no. of colonies	< 20 colonies

If the degree of positivity is different on the two slopes, the highest degree is reported.

Drug susceptibility tests

Drug susceptibility tests are undertaken against streptomycin, isoniazid, rifampicin and ethambutol on L-J medium by the proportion method.⁽⁵⁾ This method is not affected by inoculum size or viability of the cultures.

Bacterial suspension

A suspension is prepared by adding approximately 4 mg moist weight of a representative sample of the bacterial mass visualized as 2/3 loopful of 3 mm internal diameter 24 SWG wireloop into 0.2 ml of sterile distilled water in a 7 ml Bijou bottle containing 2-3 mm

The total period of contact with the alkali is 35 minutes. It has been shown that this period of contact with 4% NaOH may not be lethal to tubercle bacilli. However, in the event of longer duration of contact with the alkali, neutralize the NaOH by adding hydrochloric acid before centrifugation.^(3,4)

glass beads. This is vortexed for 30 seconds to produce a uniform suspension. To this, 3.8 ml of sterile distilled water is added to give a suspension containing approximately 1 mg/ml (S_1). From this suspension a 10-fold dilution is made by adding 0.2 ml to 1.8 ml sterile distilled water (S_2 , 10^{-1}). Three further serial dilutions 10^{-2} (S_3), 10^{-3} (S_4) and 10^{-4} (S_5) are prepared in a similar manner. One standard loopful (3 mm diameter, 27 SWG) is inoculated onto drug-free and drug-containing LJ slopes as indicated below:

Suspension	Control drug-free	S 4 mg/l	H 0.2 mg/l	R 40 mg/l	E 2 mg/l	PNB 500 mg/l
S_1 (1mg/ml)	x	x	x	x	x	x
S_2 (10^{-1})	x	x	x	x	x	
S_3 (10^{-2})	xx	x	x	x	x	
S_4 (10^{-3})	xx	x	x	x	x	
S_5 (10^{-4})	xx					

The standard strain *M.tuberculosis*, H37Rv is tested with each new batch of medium. The recommended drug concentrations are 4 mg/l for **streptomycin**, 0.2 mg/l for **isoniazid**, 40 mg/l for **rifampicin** and 2 mg/l for **ethambutol**.

Incubation and reading

Incubate the slopes at 37°C.

Read the proportion tests at 28 and 40 days.

Record growth as

+++	Confluent growth
++	More than 100 colonies
Actual number of colonies	1-99

When the number of colonies on a given dilution is less than 15, count the number of colonies with the next larger inoculum, or estimate if more than 100. (Make no attempt to estimate the number of colonies if the growth is +++)

Interpretation of tests

Interpretation of all tests is based on the 40-day readings. For each strain, express the number of organisms resistant to each drug concentration as a percentage of the number of organisms growing on the drug-free slope. Make the selection of slopes for estimating the growth on the drug-free and drug containing media in the following order of preference.

Drug-free slope

- (1) 20-70 colonies.
- (2) 5-19 colonies.
- (3) More than 70 colonies.

Drug-containing slopes

- (1) 1-5 colonies or more in the same row as the control slope.
- (2) 5-100 colonies in the row nearest to the control slope.
- (3) 1-4 colonies in the same row, or the row nearest to the control slope.
- (4) No colonies in the row farthest (including the same row as the control slope).
- (5) More than 100 colonies, if there are no acceptable counts.

Definitions of resistance - proportion method

Drug	Concentration (mg/l)	Proportion
Streptomycin	4	1% OR MORE
Isoniazid	0.2	1% or more
Rifampicin	40	1% or more
Ethambutol	2	1% or more

Calculation of proportions - an illustration

Suspension	Drug-free medium		Concentrations of streptomycin	
			4 mg/l	8 mg/l
S1	3+	(50,000)	2+	2
S2	3+	(5,000)	2+	<u>26</u>
S3	2+ 2+	(500)	<u>32</u>	3
S4	46 54	(50)	4	1
S5	4 6			
Proportion resistant			6.4%	0.52%

Preparation of drug containing media

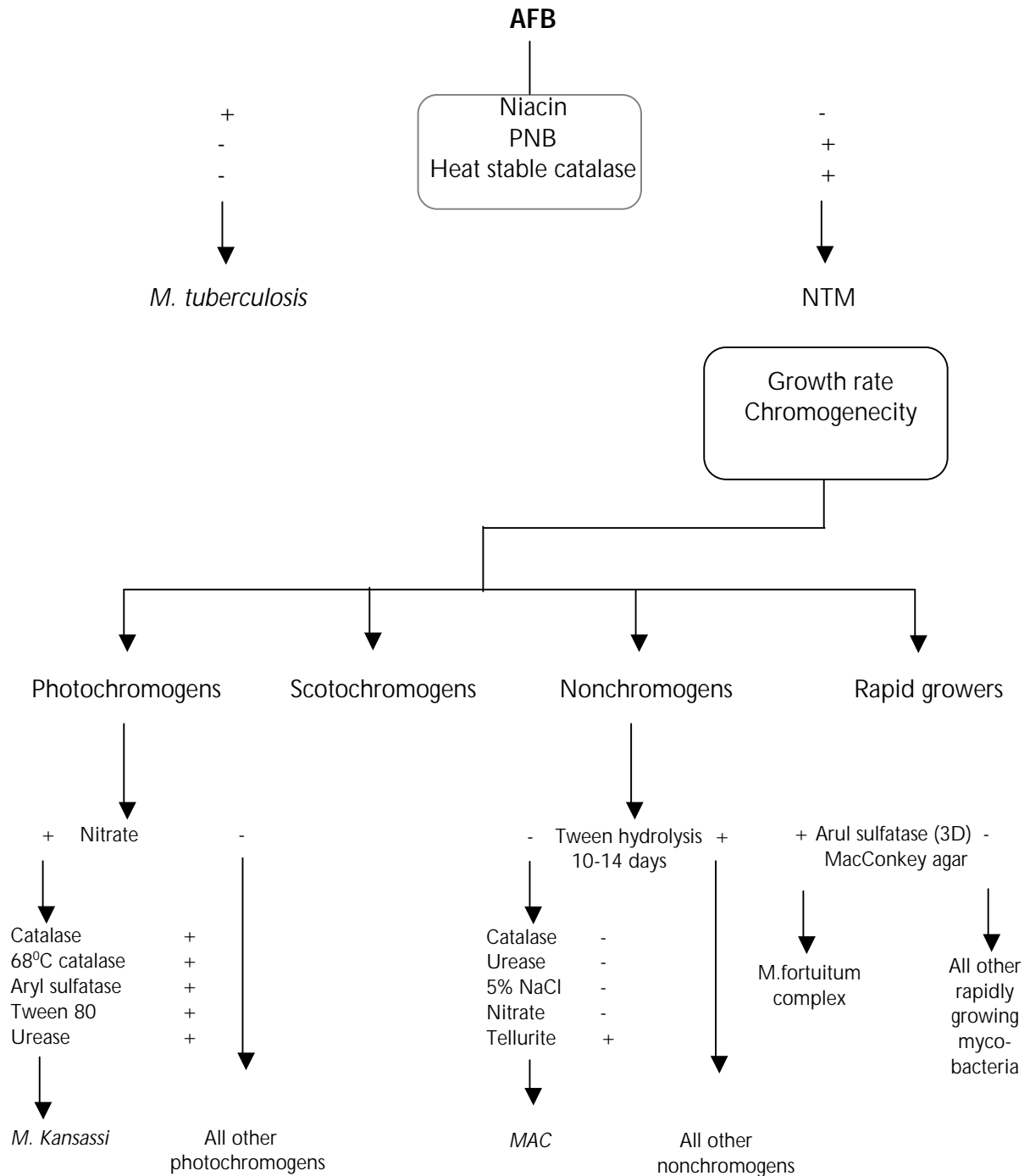
Drug-containing L-J slopes are made by adding appropriate amounts of drugs aseptically to L-J fluid before inspissation. A stock solution of the drugs is prepared to contain 10,000 mg/l in sterile distilled water for streptomycin, isoniazid and ethambutol; rifampicin is dissolved in dimethyl formamide. The sterile solutions of isoniazid and ethambutol are sterilized by membrane filtration. Suitable working dilutions are made in sterile distilled water and added to the L-J fluid, dispensed in 6-ml amounts and inspissated once.

Identification

The identification of strains isolated as belonging to *M.tuberculosis* complex or Non-Tuberculous mycobacteria (NTM) can be ascertained by performing a few simple tests *i.e.* susceptibility to p-nitrobenzoic acid (PNB), niacin production test and catalase activity of at 68°C /pH7 and nitrate reduction test, besides looking at their growth rate, temperature requirement and the morphological appearance (Figure 3).

Figure 3. **Speciation of Mycobacteria**

Work flow of speciation of Mycobacteria



Identification of mycobacteria ⁽⁶⁾

Differentiation between *Mycobacterium tuberculosis* and Non-Tuberculous Mycobacterium (NTM).

Characteristic	M. tuberculosis	NTM
Growth rate	Slow grower	Slow/Rapid grower
Temperature	37°C	25°C - 45°C
Colony morphology	Dry, rough	Dry
Colony on solid media	Eugonic	Dysgonic
Colour of colony	Buff	Yellow, orange or creamy
Emulsify	Difficult	Easy
Cord formation	+	-
Niacin test	+	-
Nitrate reduction test	+	+ / -
at 68°C Catalase test	-	+
Growth on p-nitrobenzoic acid (PNB) 500 µg/ml	-	+

Identification of non-tuberculous mycobacteria

Mycobacteria have been classified as obligate pathogens, facultative or opportunistic pathogens, or free living saprophytes. Obligate pathogens are species that do not appear to multiply outside their hosts and include *M.tuberculosis*, *M.bovis*, *M.africanum*, *M.asiaticum*, *M.farcinogens*, *M.haemophilum*, *M.leprae*, *M.malmoense*, *M.microti*, *M.paratuberculosis*, *M.shimodei*, *M.simiae* and *M.szulgai*.

The habitat of majority of the mycobacteria responsible for NTM disease is primarily environmental in existence and most of them exhibit inherent resistance to anti-TB drugs, making treatment difficult. The NTM strains may be broadly divided into those as the ones which are potentially pathogenic and those which are saprophytic in existence and usually are nonpathogenic, although they may be responsible for disease under exceptional situations. Hence, criteria for diagnosis of pulmonary disease due to NTM are rather more stringent. The American Thoracic Society has given the following criteria for diagnosis of disease due to NTM:

- (1) Evidence such as infiltrate, visible on chest skiagram of disease, the cause of which has not been determined by careful clinical and laboratory studies; and
- (2) Either (a) isolation of the same strain of mycobacteria repeatedly, usually in the absence of other pathogens, or (b) isolation of the same strain of mycobacterium from a closed lesion from which the specimen has been collected and handled under sterile conditions, for example, an abscess or biopsy tissue. Occasional isolation of these organisms from sputum, throat washings and gastric aspirates in the absence of related disease may occur and are not considered significant diagnostically.

Table 1. Most frequently identified non-tuberculous mycobacteria in medical laboratories and their presence in the environment⁽⁷⁾

Presence in Environment	Potentially pathogenic	Usually saprophytic
Natural environment		
Frequent	MAC, * <i>M.scrofulaceum</i> , <i>M.fortuitum</i>	<i>M.gordonae</i> , <i>M.terrae</i> <i>M.nonchromogenicum</i>
Infrequent	<i>M.avium</i> , <i>M.intracellulare</i> , <i>M.chelonae</i> , <i>M.malmoense</i> , <i>M.simiae</i> , <i>M.asiaticum</i> , <i>M.marinum</i>	<i>M.flavescens</i> , <i>M.vaccae</i> <i>M.aurum</i> , <i>M.gastri</i> , <i>M.smegmatis</i> , <i>M.thermoresistibile</i>
Artificial environment		
Frequent	<i>M.kansasii</i> , <i>M.xenopi</i> , <i>M.avium</i> , MAC*	<i>M.gordonae</i>
Infrequent	<i>M.marinum</i>	
Not (yet) found in Environment	<i>M.ulcerans</i> , <i>M.haemophilum</i> , <i>M.genaves</i> , <i>M.szulgai</i> , <i>M.shimoidei</i> , <i>M.celatum</i>	<i>M.triviale</i>

* The *M.avium* complex (MAC) includes several environmental species that resemble *M.avium* and *M.intracellulare* or that have intermediate characteristics common to these species.

Before the AIDS pandemic, non-tuberculous mycobacteria rarely caused serious illness, even in the immunocompromised. The prolonged immunosuppression of the cell mediated immune system caused by the HIV provided the opportunity for these relatively avirulent organisms to cause disease. *M.avium* complex (MAC) was recognized in the Western countries in the early AIDS pandemic as a cause of serious disseminated infection and is now the most common cause of systemic bacterial infection in AIDS, affecting 15%-40% of patients. Besides MAC, many other nontuberculous mycobacteria such as *M.kansasii*, *M.celatum*, *M.chelonei*, *M.fortuitum*, *M.genavense*, *M.gordonae*, *M.haemophilum*, *M.malmoense*, *M.marinum*, *M.scrofulaceum*, *M.simiae* and *M.xenopi* have been reported to cause disease.

***Mycobacterium avium* complex (MAC)**

Prior to the AIDS epidemic, MAC infection or disseminated MAC infection was extremely rare. On the other hand, MAC contributes significantly to mortality and morbidity nowadays. MAC infections are environmentally acquired diseases and the route of infections can be by inhalation, ingestion or direct contact with damaged skin.

Natural habitat

The MAC bacilli are environmental mycobacteria which are recovered from coastal waters, marine waters, soils and aerosols collected from acid water swamps. High MAC numbers correlate with warmer temperature, low pH, low dissolve oxygen, high soluble zinc and high humic acid. Such waters, soils and aerosols represent major environmental sources likely to be connected with the higher incidence of human infection.

Clinical manifestations of MAC infection in AIDS patients

Between 1981 and 1987, 2 269 cases of disseminated mycobacteriosis in patients with AIDS were reported to the Centre for Disease Control, Atlanta, USA. In 96% of cases, infection was caused by *M. avium* complex. These patients survived a shorter time (median 7.4 months) than did other AIDS patients (median 13.3 months; $p < 0.0001$.) It was concluded that disseminated mycobacteriosis was acquired by unavoidable environmental exposure.

Severe immunosuppression is the most significant risk factor for disseminated MAC. It rarely occurs in patients with CD4 counts above $100/\text{mm}^3$.

MAC infection is often under-diagnosed because of its non-specific symptoms and signs such as fever, night sweats, weight loss, nausea, vomiting, intractable crampy abdominal pain, diarrhoea and malaise. The onset of illness is usually insidious, and symptoms are often present for many weeks before the diagnosis is established. Accompanying clinical signs and laboratory abnormalities frequently included cachexia, hepatosplenomegaly, anaemia, neutropenia and elevation of alkaline phosphatase level.

Organs most commonly affected by MAC are the liver, spleen, bowel and bone marrow. MAC usually originates from the gastrointestinal tract and eventually disseminates along the reticulo-endothelial system which predominately affects the lymph nodes, liver and spleen. Endobronchial lesions and focal pneumonia may occur with or without bacteraemia. MAC may occasionally produce skin lesions. Its clinical presentation on the skin may be in the form of papules, nodules, or ulcers at the antecubital fossa and perianal area. The untreated disseminated MAC has a short survival, with a mean of 5.6 ± 1.1 months (median 4 months).

Essential laboratory investigations and their interpretations

Microscopy

A presumptive diagnosis of MAC infection can be made quickly by demonstration of acid fast bacilli (AFB) in smears from tissue *e.g.* skin, bone marrow, lymph node, liver biopsy or buffy coat of blood in patients with clinical pictures mentioned above, but definite diagnosis still requires culture confirmation.

Culture

The definite diagnosis of disseminated MAC infection is usually made by isolation of organisms from blood or bone marrow cultures. Cultures of MAC require 2 to 6 weeks to become positive. The BACTEC 9240 system is faster but still requires a minimum of 8-10 days to become positive and may require up to four weeks in samples with low colony counts.

Haemoculture for MAC

Recovery of mycobacteria from blood, especially in cases of disseminated disease associated with AIDS, presents some unique problems. Recently, the lysis-centrifugation

technique with the DuPont Isolator tube has successfully recovered *M.chelonae*, *M.avium* complex and *M.tuberculosis* from blood. A 10 ml volume of blood is added to the Isolator tube; the tube is inverted several times to mix the contents, and then it is allowed to stand for one hour to lyse the blood cells. After lysis, the tube is centrifuged at 3000g or more to concentrate the material into 1.5 – 2.0 ml of sediment, or of which is spread over the surface of several tubes or plates of isolation media (e.g. 7H10, 7H11, L-J). By using appropriate dilutions of sediment (or diluting lysed specimens prior to centrifugation), it is also possible to quantify the extent of the bacteremia.

Other rapid methods for culture

Today, there are few rapid methods for the culture and measurement of the susceptibility of mycobacteria. These include microcolony detection on solid media, the Septi-Check AFB method, mycobacterial growth indicator tube (MGIT) system, BACTEC radiometric method, and MB/Bact mycobacteria detection system.

Laboratory methods based on DNA technology

Nucleic acid probes with non-isotopic detection systems are available for the *M. tuberculosis* complex, *M. avium*, *M. intracellulare*, *M. avium* complex, *M. kansasii* and *M. gordonae* and more will become sooner available. These can be used to identify organisms directly from clinical samples, cultures from conventional media and from radiometric vials. However, these tests are expensive. Since the use of these techniques requires higher technical expertise and training, their implementation is possible only in a reference laboratory.

Quality assurance programme

Quality control procedures should be performed on a regular and periodic basis in the mycobacteriology laboratory to assure reproducibility and reliability of laboratory results.

Sputum smear Microscopy

This remains the basis for diagnosis of TB in most SEAR countries. The importance of correct readings of sputum smears at the local level, where the diagnosis is usually made, is critical. The IUATLD currently recommends that a systematic sample of sputum smear microscopy specimens be selected for review. The sample should include both positive and negative specimen smears, and the slides should be re-read by a second individual who did not perform the initial specimen slide reading; the second individual should not know the result of the first reading (blinded re-reading).¹⁶

Culture

Records must be kept on all “homemade” culture media and should be checked for sterility and sensitivity. The latter must be carried out by using the standard suspension of *M. tuberculosis* H37Rv.

Likewise QC must be ensured for procedures such as digestion and decontamination, media, reagents and biochemical tests employed for the isolation and identification of mycobacteria.

Drug susceptibility

Besides internal quality control (IQC) procedures, each centre must participate in an external quality assessment scheme (EQAS) adhering to the norms prescribed by WHO in the Global Drug Resistance Surveillance (DRS) programme.

Storage of cultures

From each patient, at least one culture must be expanded, tested for its purity and then stored at -70°C till the end of the study.

Grade of TB laboratory diagnostic procedures to be performed at different levels

Level I: Peripheral	These are usually single room or parts of another laboratory at the primary health care or local hospital level. Tuberculosis investigation is restricted to collection and smear examination and despatch of specimens to a higher level laboratory for culture.
Level II: Intermediate	These laboratories may be in larger hospitals, such as district hospitals and be part of pathology laboratory complex. The work includes examination of direct smear and, in some culturing specimens, but the cultures are usually sent to a higher grade laboratory for incubation and further tests.
Level III: Central	These more specialized laboratories examine direct smears and culture specimens for mycobacteria. Some may do no more than this and send the cultures to other TB level III laboratories. Others continue with the identification of the mycobacteria to species level and do antituberculosis drug susceptibility tests. The highest grade of TB level III laboratories usually do research and function as training centres and as reference laboratories.

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Appendix

Preparation of stain

Preparation of 1% carbol fuchsin

- (1) Weigh 5 grams of basic fuchsin dye in a balance and transfer it to a 250 ml Erlenmeyer glass flask.
- (2) Add 50 ml of methylated spirit and shake to dissolve the dye.
- (3) Heat 25 grams of phenol until it melts and add to the above solution.
- (4) Heat the flask containing basic fuchsin dye dissolved in spirit and phenol in a water bath at about 60°C. **Do not heat directly on a flame.**
- (5) Transfer the contents into a 500 ml measuring cylinder.
- (6) Add distilled water to make up a final volume of 500 ml.
- (7) Pour the solution through filter paper (Whatman No.1) and store filtered solution in a glass bottle. Label the bottle as 1% carbol fuchsin with the date of preparation.

Preparation of 25% sulphuric acid

- (1) Pour 375 ml of distilled water into a 1 litre glass flask.
- (2) Measure 125 ml of concentrated sulphuric acid and transfer it slowly into the flask containing water.
Always add acid to water. Never add water to acid.
- (3) Store the sulphuric acid solution in a labelled glass bottle.

Preparation of 0.1% methylene blue solution

- (1) Weigh 0.5 grams of methylene blue and transfer to a 1 litre glass flask.
- (2) Add 500 ml of distilled water.
- (3) Shake well to dissolve.
- (4) Store in a glass bottle with the label showing name of the reagent and date of preparation.

Sodium hydroxide

Dissolve 40g sodium hydroxide in 1,000 ml distilled water, and distribute approximately 200 ml of the solution in 250 ml gauze-covered cotton wool-stoppered conical flasks and sterilize by autoclaving. Keep at 37°C until used.

Preparation of culture media

Lowenstein Jensen Medium-Drug free

Salt solution with malachite green (SSMG)

Potassium dihydrogen orthophosphate, 0.4%	14.4 g
Magnesium sulphate, anhydrous, 0.04%	1.44 g
Magnesium citrate, 0.1%	3.6 g
L-asparagine, 0.6%	21.6 g
Glycerol, 2%	72 ml
Malachite green, 2% solution	120 ml
Distilled water, to	3,600 ml

Distribute in 600 ml amounts, autoclave and store in refrigerator at 2-8°C.

Complete medium

To 1 litre of egg fluid, add 600 ml of SSMG, mix well and distribute in approx. 6 ml amounts. Inspissate at 85-90°C for 50 minutes. Re-inspissate after overnight storage at room temperature.

Lowenstein-Jensen with sodium pyruvate

For this prepare the salt solutions with malachite green as above *omitting glycerol*. Proceed as above and to every 1 600 ml of complete medium, add 8 gm sodium pyruvate (0.5 %). Distribute and inspissate.

Susceptibility to PNB

This test is included along with the DS tests. PNB is incorporated in L-J medium at a concentration of 500 mg/l and inoculated with one loopful of a standard suspension. *M.tuberculosis* complex is inhibited by PNB while all other mycobacteria are resistant .

Nitrate reduction tests

Classical method with liquid reagents

Media and supplies

- Sodium nitrate (NaNO_3)
- Monopotassium phosphate (KH_2PO_4)
- Disodium phosphate ($\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$)
- Hydrochloric acid (HCl)
- Sulphanilamide

- N-naphthylethylenediamine dihydrochloride
- Distilled water
- Zinc dust
- Screwcap test tubes , 16x125 mm
- Water bath or constant-temperature block heater , 37°C

Preparations

Substrate

To prepare 0.01 M sodium nitrate in 0.022 M phosphate buffer, pH 7.0 dissolve in order the following chemicals in 100 ml of distilled water : 0.085 g NaNO₃, 0.117 g KH₂PO₄, 0.485 g Na₂HPO₄.12H₂O. Sterilize by autoclaving.

Reagent#1

Carefully add 50.0 ml of concentrated HCl to 50.0 ml of distilled water. **Never add water to acid.**

Reagent#2

Dissolve 0.2 g of sulphfanilamide in 100.0 ml of distilled water.

Reagent#3

Dissolve 0.1 g of N-naphthylethylenediamine dihydrochloride in 100.0 ml of distilled water.

Store the substrate and reagents in the dark at 5°C. Discard the reagents if the color changes or a precipitate forms and prepare afresh.

Controls

Positive = *M. tuberculosis* (3+ to 5+).

Negative = *M. bovis* (BCG) or *M. intracellulare*.

Negative = reagents without organisms.

Procedure

- (1) Add 0.2 ml of sterile distilled water to a 16 x 125 mm screwcap tube.
- (2) Use a sterile spade or applicator stick to emulsify in the water two spadesful of growth from a four week old culture on Lowenstien -Jensen or egg-base medium.
- (3) Add 2.0 ml of the NaNO₃ substrate to the tube.
- (4) Shake by hand and incubate upright for two hours in a 37°C water bath.
- (5) Remove from the water bath.

- (6) Add one drop reagent # 1.
- (7) Add two drop reagent # 2.
- (8) Add two drop reagent # 3.
- (9) Examine immediately for a pink to red color.

Results and interpretation

Positive = May range from pale pink to deep red

Negative = No color.

Niacin production test

Autoclave (15 lb for 30 minutes) a control drug-free slope after the final reading of the sensitivity or identification test. Add 0.5 ml distilled water to the culture bottle if no condensation water is present before autoclaving.

Work in a protection cabinet.

To a 75 x 12 mm test tube add 0.25 ml of the culture extract followed by 0.25 ml of 1.5% o- toluidine solution and an equal volume of 10% cyanogen bromide solution. Shake by hand to mix and read the result within one minute. Pink to red colour of the precipitate indicates positive, white precipitate indicates negative.

Strains of *M.tuberculosis* are almost always niacin-positive. All other types are negative.

Reagents

- (1) Approximately 10% solution of cyanogen bromide in distilled water. (A saturated solution of cyanogen bromide is approximately 10%). Store in a brown bottle at 4°C.
- (2) 1.5% o-tolidine in ethyl alcohol, freshly prepared just before use.

Stability of catalase at pH 7.0/68°C

Reagents

- (1) M/15 phosphate buffer, pH 7.0: Sterilise in 20ml. amounts in universal containers.
- (2) Hydrogen peroxide, 30% AR.
- (3) Tween-80, 10% solution.

Method

- (1) With a sterile 1ml pipette, add 0.5 ml of buffer to sterile screw-capped test tubes.
- (2) Emulsify a loopful of an actively growing culture into each tube of buffer.

- (3) Place the tubes with the emulsified suspension in a water bath maintained at 68°C for 20 minutes. The time and temperature are critical.
- (4) Remove from the water bath and cool to room temperature.
- (5) Add to each tube 0.5 ml of the peroxide-tween mixture inside a safety cabinet.
- (6) Observe for bubble formation on the surface of the liquid, without shaking. Hold tubes for 20 minutes before discarding.

Report as positive, if bubbles are observed and as negative if no bubbles are seen. *M. tuberculosis* are negative for heat stable catalase.

Controls

Use uninoculated buffer as negative control.

4. Standard Operating Procedures for the Laboratory Diagnosis of other Bacterial Infections in HIV/AIDS Patients

Introduction

In HIV-infected patients, the bacterial infection is one of the causes of severe complications. AIDS patients can also be infected by the pathogenic bacteria that infect normal persons. Moreover, these patients may be infected by the opportunistic bacteria. Examples of the most common opportunistic bacteria in AIDS patients are *Salmonella*, and *Rhodococcus equi*.

Clinical association and diagnosis

Salmonellosis is a disease most commonly caused by ingestion of food, water or milk contaminated with *Salmonella*. Three types of infections are gastroenteritis, bacteraemia or septicemia and enteric fever. Routine culture using selective and differential culture media usually is sufficient for isolating *Salmonella*. The identification can be done by conventional biochemical tests.

Rhodococcus equi, an aerobic gram-positive coccobacilli, formerly known as *Corynebacterium equi*, was considered as an extremely rare opportunistic bacterial pathogen in man. In recent years, there has been a dramatic increase in the number of reported isolations in HIV/AIDS patients. Patients with rhodococcus present with bronchopneumoniae, bacteraemia, skin infection, endophthalmitis, peritonitis, catheter-associated sepsis and prostatic abscess. Lethality is high, particularly in HIV-infected patients. Laboratory diagnosis is made by Gram stain, modified acid fast stain and culture from the specimen.

Safety considerations

Specimen collection	<ul style="list-style-type: none">• Universal precautions and aseptic technique to be followed.
Specimen transport and Storage	<ul style="list-style-type: none">• Sterile leak proof and appropriate container in a sealed plastic bag.
Specimen processing	<ul style="list-style-type: none">• Universal precaution to be followed.• At least biosafety level II with good laboratory practice.

The above guidelines should be supplemented with recommendations from the local health hazard and risk assessment committee.

Specimen collections

Optimum time of specimen collection	<ul style="list-style-type: none"> Ideally before initiation of antimicrobial therapy.
Correct specimen type	<ul style="list-style-type: none"> Depends upon the clinical criteria and the organism suspected e.g. haemoculture, sputum, stool or urine. Appropriate specimen from the site of infection in appropriate container and adequate amount.

Specimen transport and storage

Time between specimen collection and processing.	<ul style="list-style-type: none"> Ideally the specimen should reach the laboratory within 30 minutes of collection. Urine which arrives at the laboratory more than two hours after collection is not suitable for culture and should be rejected. Specimens in the transport medium should be sent to the laboratory as fast as possible otherwise the pathogenic bacteria will be overgrown by normal flora. Specimens should be processed promptly after receipt.
Storage	<ul style="list-style-type: none"> Any specimen that cannot be sent to the laboratory within 30 minutes or cannot be processed immediately should be stored in the refrigerator or freezer (except CSF, body fluids and haemoculture) or put in an appropriate transport medium where applicable.

Specimen processing

Proper documentation upon receipt of specimen	<ul style="list-style-type: none"> Record the correctness of the specimen on the requisition form. Maintenance of laboratory records with lab number.
Initial processing	<ul style="list-style-type: none"> Depends upon the types of specimen collected and organism suspected. Haemoculture (blood culture) should be incubated at 35°C immediately. CSF and other body fluids should be centrifuged at 2500g for 15 minutes. The sediment is used for culture and staining.
Microscopy	<ul style="list-style-type: none"> Perform staining as required. Gram stain is a common stain for bacteria Acid fast stain for <i>Mycobacterium</i> Modified acid fast stain for <i>Rhodococcus</i> and <i>Nocardia</i>
Reporting procedure	<ul style="list-style-type: none"> Accuracy and timeliness of reporting procedure to be practised. Report results by telephone in critical care patient. Written report Staining: report one day Culture and susceptibility: report 3-5 days (Report susceptibility test as clinically indicated)

Preservation of specimens	<ul style="list-style-type: none"> • Specimen should be kept for confirmation wherever applicable.
Disposal of specimen	<ul style="list-style-type: none"> • All specimens must be autoclaved or disinfected before being discarded. • Applicator sticks and gauge pieces should be incinerated. • All used glassware should be put into the disinfectant pan for at least one hour before washing.

Isolation and identification of *Rhodococcus equi*

Isolation

The isolation of *Rhodococcus* can be performed by plating the specimen onto blood agar medium. Incubate the plates at 35°C in ambient air. On primary culture, the colonies are usually visible after 24-48 hours and attain a diameter of 1-2 mm. Older colonies tend to become larger and mucoid with a characteristic salmon-colored pigment. Appearance of the pigment may be delayed.

Identification

Rhodococcus can be presumptively identified via the staining technique. Any specimen that shows gram-positive pleomorphic coccobacilli and stained red with modified acid fast stain may be suspected as *Rhodococcus*.

The characteristic colonies that are gram-positive pleomorphic coccobacilli or diphtheroid-like should be subcultured. The pure culture of the suspected organism can be identified by conventional biochemical tests.

The modified acid fast staining from the colonies usually gives negative results.

Antimicrobial susceptibility test

According to the NCCLS, there still have no standard zone interpretation using the Kirby-Bauer susceptibility test for this organism. In cases of clinical importance, the MIC technique may be performed where applicable.

Isolation and identification of *Salmonella*

Isolation

The organisms belonging to genus *Salmonella* are gram-negative, non acid fast, non capsulated and non-sporing bacilli, which measure approximately 2-4 µm x 0.6 µm. Almost all species of *Salmonella* are motile. The organisms grow rapidly on ordinary media and optimum temperature of growth is 37°C. The *Salmonella* spp. other than *S. typhi* and *S. paratyphi* have been reported as opportunistic bacterial infections in HIV/AIDS patients.

Identification

The growth of *Salmonella* on MacConkey agar is pale yellow (non lactose fermenting) colonies, 2-3 mm in diameter, moist, circular and smooth convex surface. These are catalase positive and oxidase negative. The organisms can be identified by conventional biochemical tests. An organism identified as *Salmonella*, on the basis of biochemical reactions, can be confirmed with slide agglutination test, using polyvalent 'O' and polyvalent 'H' antisera against salmonellae. To perform this test, one drop each of normal saline is placed at two different sites on a clean glass slide. A loopful of biochemically suspect colony of *Salmonella* is emulsified in both. One of these is kept as control and another is tested first with polyvalent 'O' antiserum against *Salmonella*. If this gives visible agglutination within two minutes, the process is repeated with polyvalent 'H' antiserum. If the serogroup is to be determined, the test should be further performed with monovalent 'O' antisera against *Salmonella*.

Antimicrobial susceptibility test

The emergence and spread of *Salmonella* resistant to multiple antibiotics has assumed a massive proportion. For this reason, conventional antibiotics such as chloramphenicol, ampicillin and trimethoprim sulfamethoxazole can no longer be considered as the first line drugs in the treatment. Each and every isolate of *Salmonella* should be subjected to antimicrobial susceptibility test using the standard method. This will not only help in the management of the patient more efficiently but will also help in generating the surveillance data which will be useful to formulate antibiotic policy in a given hospital.

Nocardiosis

Nocardiosis is an acute or chronic, suppurative (less commonly granulomatous) disease caused by the soil-inhabiting aerobic actinomycetes, *Nocardia asteroides*, *Nocardia brasiliensis*, and *Nocardia otitidiscaviarum*. There are three distinct clinical syndromes that may evolve : (1) primary cutaneous, (2) primary subcutaneous and (3) primary pulmonary and systemic.

Natural habitat

Gordon and Hagen first isolated *N. asteroides* from soil by the paraffin technique. These findings have been confirmed by other investigators, and it appears that the organism has a worldwide distribution. *N. brasiliensis* and *N. otitidiscaviarum* have also been recovered from soil.

Several species of the genus *Nocardia*, viz. *N. asteroides*, *N. brasiliensis*, *N. caviae* and perhaps *N. farcinica*, are valid etiologic agents of the clinical disease nocardiosis in humans. *N. brasiliensis* is more virulent than *N. asteroides* or *N. otitidiscaviarum*. It is able to cause infection readily in experimental animals as well as systemic disease in normal patients.

Clinical manifestation

Nocardiosis has appeared in multiple case reports, particularly in relation to its association with other diseases and with the use of antileukaemic drugs, cytotoxins, immune depressants, corticosteroids, and as a complication of the AIDS. The opportunistic nature of the infection is now emphasized and there are approximately 1 000 cases each year in the United States. *N. asteroides* accounts for about 90% of cases, *N. brasiliensis* for nearly 7 % and *N. otitidiscaviarum* for less than 3 %.

With rare exceptions, nocardiosis is a pulmonary disease of respiratory origin that results from the inhalation of spores. Cerebral nocardiosis associated with pulmonary disease is frequently reported (about 27 per cent of pulmonary infection involves the central nervous system also), and there is conclusive evidence of hematogenous spread. Primary or secondary lesions in the gastrointestinal tract at a site of pre-existing mucosal ulceration and, rarely, appendiceal involvement may result from ingestion of contaminated material or sputa from infected lungs.

Essential laboratory investigations and their interpretation

Microscopy

Sputum, pus, tissue material and so forth can be examined for *N. asteroides*. The materials to be examined may be digested, then concentrated by centrifugation. Gram's stain of the material will show long, sinuous, branching, gram-positive filaments and fragmented bacillary bodies. The branching tends to be at long intervals and at right angles to the main axis of the mycelium. A modified acid-fast stain will show beaded or fragmented acid-fast bacillary forms. This distinguishes the organism from *Actinomyces*, but it may be confused with the tubercle bacillus. Both *N. asteroides* and *N. brasiliensis* are acid-fast ; most other actinomycetes are not. Rarely, *N. asteroides* aggregates into a soft pseudogranule, whereas *N. brasiliensis* regularly forms true granules.

Culture

N. asteroides grows readily on ordinary laboratory media without antibiotics. It is aerobic but it grows out equally well under anaerobic conditions. Its optimal growth temperature is 37°C. Both *N. asteroides* and *N. brasiliensis* develop slowly on routine media, particularly on Sabouraud's agar. By two to three weeks, they attain a diameter of 5 to 10 mm. The colonies are waxy, folded and heaped at first. They may later develop areas of downy or tufted aerial mycelia. The whole surface may become dry and powdery. A musty, dirt like odor is sometimes present. The colour range included orange, pink, white, buff, brown, lavender and salmon.

Serological tests

Serological procedures have been tried on sera from patients with nocardiosis, but no consistency of response has been found. Circulating antibodies have been reported in patients with nocardiosis, but their role in defense has not been assessed. Complement fixing and agglutinating antibodies have been reported. A fluorescent antibody technique

for specific identification of *N. asteroides* has been attempted, but sensitivity was only 20 per cent or above. At the present time, there is no reliable diagnostic or prognostic serological procedure for the disease.

Isolation and identification of *Nocardia*

Isolation

Nocardia, a member of the actinomycetes, is gram-positive branching filamentous, often with a bead appearance. *Nocardia* spp. are able to grow on routine laboratory media such as sheep blood agar. However, because *Nocardia* spp. grow slowly and require a minimum of 48 to 72 hours of incubation, it may be overgrown by other normal flora present in contaminated specimens. A solid medium that uses paraffin as the sole source of carbon has been effective for isolating *Nocardia* spp. from contaminated clinical specimens. *Nocardia* spp. also grow well on Sabouraud dextrose agar but are inhibited by chloramphenicol. Thus, chloramphenicol should not be added into the medium. Although *Nocardia* spp. grow at 35°C, some strains grow well at 30°C. Plates should be incubated for 2 to 3 weeks. The colonial appearance of *Nocardia* spp. are extremely variable; some isolates are beta-hemolytic on sheep blood agar. Some are wrinkled; often dry, chalky-white appearance to orange-tan pigment.

Identification

The Gram stain from suspected colony should be performed. If Gram-stain morphology is suggestive of a possible *Nocardia* spp. ie. Gram-positive branching, fine, delicate filaments with fragmentation, a Ziehl-Neelsen acid-fast stain should first be performed, followed by a modified acid-fast stain. If the modified acid-fast stain results are positive, the isolate may be reported, preliminarily, as *Nocardia* spp. If acid-fast stain-negative, these organisms are still not completely ruled out because of the variability of acid-fastness among isolates belonging to this group. To confirm the identification, the isolate should be referred to a reference laboratory.

Antimicrobial susceptibility test

Although various methods are available, the antimicrobial susceptibility testing of *Nocardia* spp. remains problematic. Some of the problems include the lack of standardized, validated methods, lack of correlation of in vital susceptibility testing results with clinical outcome, and the inability to achieve a uniform suspension of organisms for testing for all strains. Nevertheless, antimicrobial susceptibility testing should be performed on clinically significant isolates and also should be referred to a reference laboratory.

References

1. Forbes BA, Sahm DF, Weissfield AS (1998) Diagnostic Microbiology, Tenth edition.
2. Beaman BL, Saubolle MA, and Wallace RJ. (1995) *Nocardia*, *Rhodococcus*, *Streptomyces*, *Oerskovia*, and Other Aerobic Actinomycetes of Medical Importance. p. 379-399. In Murray PR, Baron EJ, Pfaller MA, Tenover FC, and Tenover FC. (ed.) Manual of Clinical Microbiology Sixth edition. American Society for Microbiology, Washington.

5. Standard Operating Procedures for the Laboratory Diagnosis of Common Fungal Opportunistic Infections in HIV/AIDS Patients

Introduction

Opportunistic fungus infections are increasing with the unprecedented increase in number of immuno-compromised patients in various disciplines of the health care system. The situation has become even more alarming with the current pandemic of AIDS. The commonly encountered fungal infections in HIV-positive patients are candidosis, cryptococcosis and histoplasmosis. Other fungal infections like coccidioidomycosis and *Penicilliosis marneffeii* are seen usually in geographically restricted areas. *Penicilliosis marneffeii* has been extensively reported in Thailand and South China, and more recently in North-East India. Coccidioidomycosis has never been reported in South-East Asia.

Laboratory procedures in Clinical Mycology are directed mainly towards:

- (1) Direct demonstration of the pathogenic fungi in clinical specimens;
- (2) Successful isolation of pathogenic fungi;
- (3) Supportive evidence of specific fungal infection (antigen, antibody or metabolites);
- (4) Evaluation of possible therapeutic outcome (antifungal susceptibility), and
- (5) Tracing of the source of infection (epidemiology).

Early diagnosis is essential for early effective management of the patients. There are no clinical pathognomonic signs and symptoms specific for fungal infections, especially the deep seated ones, and confident diagnosis relies heavily on a combination of microbiological, histopathological and serological evidence. Though continuous addition of newer methods takes place, introduction of sophisticated instruments in day-to-day working of the laboratory invariably increases the cost of laboratory investigations. Therefore, at the primary level, the mycologist has to fall back upon simple established standard procedures. No one single procedure may help in proper diagnosis, since each has its own limitations. Therefore, all these procedures should be incorporated in a standard mycological laboratory as far as practicable.

It is necessary that adequate amounts of appropriate specimens, properly collected and handled, are received. Therefore, these are the basic aspects that need to be addressed for proper functioning of the laboratory.

The procedures of mycological investigation that can be undertaken at the primary level are simple and no elaborate equipment is required. For advanced techniques, specimens should be referred to the better-equipped Reference Laboratories designated for this purpose.

Common opportunistic infections in AIDS patients

Candidosis

Candidosis is a common endogenous opportunistic yeast infection. Of the causative agents, the most common species is *Candida albicans*, though other non-*albicans* species are increasingly being reported.

C. albicans causes mostly superficial mucosal infection. It is the commonest fungal infection found in HIV/AIDS patients. Extensive esophageal candidosis is an AIDS defining infection. Though oral candidosis, unless very extensive, is not diagnostic of AIDS, it is of prognostic value as its presence indicates progression of the immunodeficiency.

Candida species is readily isolated on most laboratory media, of which the most commonly used and economical medium is SDA. Since common *Candida* species isolated from clinical specimen are not inhibited by antibiotics or cycloheximide, the use of media containing these antimicrobials is very helpful in the isolation of *Candida*, specially from specimens which are not sterile (e.g. skin, sputum and urine). Most strains grow well at 37°C or at room temperature (22^o-25°C).

Young colonies are white with a soft consistency; the surface and margins of the colonies are smooth, although rough surfaced strains have been described. Old colonies frequently show a fringe of submerged mycelium which appears as feathery outgrowths deep in the agar.

In a wet mount, masses of budding cells and fragments of mycelium, often with budding cells attached, are indicative of the presence of a yeast like organism. Gram stain of smears show gram-positive budding yeasts with both pseudomycelium and true mycelium. Pseudomycelium (hyphae) designate elongated cells formed from blastospores (budding cells) which elongate, but do not break off from the mother cell. These filaments are very fragile and may break apart easily during smear preparation. On the other hand, true mycelium is formed by the elongation and branching of a germ-tube produced by the mother cell. Septae are formed along the length of the mycelium.

All these forms, rare in immuno-competent patients, are seen abundantly in AIDS patients.

Clinical considerations

Clinical manifestations include:

- Oropharyngeal candidosis
- Oesophageal candidosis

- Vaginal candidosis
- Non-healing extensive skin ulcers (seen in Indian patients)
- Inter-triginous candidosis
- Disseminated candidosis (very rare)

Diagnosis

Specimens depend on the site of lesion. Usual specimens for investigation of candidosis are:

- Swabs/scraping from lesion/nail clippings
- Oesophageal brushing
- Blood for culture
- Urine (suprapubic, specially in children) and other body fluids
- CSF only in very rare cases

Since *Candida* is commensal, demonstration of a few cells or isolation from specimens such as sputum, or swabs from mucosal surfaces, has no significance. However, the presence of a large number of organisms in a fresh specimen may have some diagnostic significance.

In the blood, urine (suprapubic or collected with sterile precautions), CSF and sample from closed inflammatory foci, the presence of *Candida*, whatever the species and the number of cells, is of pathogenic significance. It is wise, however, to request repeat specimens, wherever possible, to rule out possible contamination from the skin when the specimen was obtained.

Cryptococcosis

The etiologic agent is *Cryptococcus neoformans*, the only pathogenic species of the genus *Cryptococcus*. There are five serotypes (A, B, C, D and AD), and it exists in two varieties, var *neoformans* and var *gatti*. Recently a new variety has been suggested (*grubeii*) for the serotype A. Cryptococcosis generally begins with primary pulmonary invasion. In immuno-competent individuals, it remains as inapparent subclinical infection. In immuno-suppressed patients it spreads and occasionally becomes disseminated. *C. neoformans* has a predilection for the central nervous system (CNS), and patients mostly present with symptoms of chronic meningitis. For many years, it was considered to be a rare disease; however, now it is recognized to be quite common, probably due to the fact that it is diagnosed more frequently.

In the present era of AIDS pandemic, cryptococcal meningitis has been considered as one of the AIDS defining infections by the WHO.

In SDA both at 25^o and 37^oC colonies may appear within 48 hours, but may take a longer time depending on the fungal load. The colonies are soft and creamy in texture, or mucilaginous if considerable capsular material is present. Upon repeated subcultures (for instance, for maintenance of isolates) the colonies tend to become dry as the capsule size decreases.

Clinical considerations

CNS cryptococcosis

- Onset insidious, initially symptoms are present for weeks.
- Headache with minimal or no neck rigidity.
- Fever of unknown origin.
- Drowsiness and alteration in sensorium, with advancement of disease process.
- Chronic meningitis.
- May result in cerebral cryptococcal granuloma.

Pulmonary cryptococcosis

- Often asymptomatic.
- Diagnosed mainly by extensive laboratory investigations and radiology.

Diagnosis

- Specimens should be collected according to the symptoms of the patient.
- Most common specimen is CSF; other body fluids, sputum, biopsy tissues, prostatic fluid (in case of relapse) and blood for culture are used where indicated. If the initial demonstration/isolation is from an extrapulmonary site, attempts should be made to examine CSF irrespective of clinical presentation, to rule out asymptomatic meningeal involvement.
- Diagnosis by conventional methods as described above: direct demonstration and culture. Blood culture is helpful specially in the disseminated condition.
- Serology: important adjunct:
 - Antigen detection – test of choice with very high sensitivity and specificity.
 - Antibody detection – has prognostic value rather than diagnostic value.
 - Antibody becomes positive as the patient recovers and antigen titer drops.
 - However, it is doubtful that AIDS patients ever recover sufficiently enough to produce detectable antibodies.

Histoplasmosis

Histoplasmosis is an intracellular mycotic infection of the reticuloendothelial system, caused by the inhalation of conidia from the fungus *Histoplasma capsulatum*. Approximately 95% of cases of histoplasmosis are inapparent, subclinical or benign. Five percent of the cases have chronic progressive lung disease, chronic cutaneous or systemic disease or an acute fulminating fatal systemic disease. It is being increasingly reported in HIV/AIDS patients from geographically restricted areas.

Two varieties of *H. capsulatum* are recognized in human diseases, depending on the clinical disease: var *capsulatum*, var *duboisii* (the African type).

Histoplasmosis has a world-wide distribution, though the Mississippi Ohio River valley in the USA is recognized as the major endemic region. Environmental isolation of the fungus has been made from soil enriched with excreta from chicken, starlings and bats.

H. capsulatum exhibits a thermal dimorphism by growing in living tissue or in culture at 37°C as a budding yeast like fungus, or in soil or cultures at temperatures below 30°C as a mould.

On SDA at 25°C colonies are mostly slow growing, white or buff brown, cottony with a pale brown transverse. Sometimes colony types may be glabrous or verrucose.

Microscopic morphology

- Macroconidia: Presence of characteristic large (8-25µm in diameter), round to pyriform, single-celled, thick-walled macroconidia formed on short, hyaline, undifferentiated conidiophores. Macroconidia develop late in the growth phase of the fungus.
- Characteristic tuberculate (finger-like or spiny projections) appearance of the macroconidia is described as definitive diagnostic feature of the fungus, but tubercles are not produced by all isolates of this fungus.
- Microconidia, if present, are small (2-4µm in diameter), round to pyriform and carried on short branches or directly on the sides of the hyphae.

On BHI blood agar at 37°C colonies are smooth, moist, white and yeast-like. Microscopically numerous small round to oval budding yeast-like cells, 3-4 x 2-3 µm in size are observed.

Traditionally, positive identification of *H. capsulatum* required

- (a) Conversion of the mould form to the yeast phase by growth at 37°C on enriched media in the presence of adequate moisture, and reversion to the mould form at 25°C.
- (b) Mouse pathogenicity testing (for conversion of mould to yeast phase) at the level of the Reference Laboratory.

However, culture identification by the exoantigen test is now the method of choice.

Clinical considerations

- All stages of this disease may mimic tuberculosis.
- Since this intracellular pathogen resides in macrophages, in a suspected case, the ideal specimen for direct demonstration and culture is bone marrow, preferably inoculated in the medium at the bedside immediately after collection from the patient.

Penicilliosis marneffeii

This infection is caused by the dimorphic fungus *Penicillium marneffeii*. It is endemic in South-East Asia and the southern region of China. It was initially reported from particular geographical areas like Thailand, but has now been reported from other parts of South-East Asia as well, and European countries where the patients have a history of travel to the endemic area. This has recently been isolated from India (Manipur) also. Bamboo rats are

considered to be the reservoirs of this disease. This is one of the important AIDS defining infections.

P. marneffei exhibits thermal dimorphism by growing in living tissue or in culture at 37°C as a yeast-like fungus, or in culture at below 30°C as a mould. On SDA at 25°C colonies are fast growing, suede like to downy, white with yellowish green conidial heads. Colonies become greyish-pink to brown with age and produce a diffusible brownish-red to wine-red pigment. Conidiophores are hyaline, smooth-walled and bear terminal verticils of 3-5 metulae, each bearing 3-5 phialides. Conidia are globose to subglobose, 2-3µm in diameter, smooth-walled and are produced in basipetal succession from the phialides.

On BHI blood agar at 37°C colonies are rough, glabrous, tan-coloured and yeast-like. Microscopically, the yeast cells are spherical to ellipsoidal, 2-6µm in diameter, and divide by fission rather than by budding. Numerous short hyphal elements and elongated sausage-shaped forms are also seen.

Tissue sections show small, oval to elliptical yeast like cells, 3µm in diameter, either packed within histiocytes or macrophages, or scattered throughout the tissue. Occasional, large, elongated, sausage-shaped cells, up to 8µm long, with distinctive septa may be present. Tissue sections need to be seen by GMS stain to clearly see the yeast like cells, which are often difficult to observe in H&E preparations.

Clinical considerations

Various types of manifestations include:

- Fever of unknown origin;
- Generalised lymphadenopathy;
- Hepatomegaly;
- Pneumonitis, and
- Skin lesion simulating molluscum contagiosum.

Diagnosis

Specimen: Bone marrow, blood (for culture and serology), sputum, BAL, skin biopsy/touch smear.

Giemsa stain shows characteristic intracellular (neutrophils or tissue histiocytes) round to oval yeast like cells, with an eccentric or central dot with occasional central septation. Elongated sausage-shaped extracellular forms are also seen. This has to be confirmed by special staining techniques, such as GMS, to be done at the level of the Reference Laboratories.

Direct immunofluorescence test is the test of choice for specific diagnosis; this would be done at the level of Reference Laboratories, since it requires a fluorescent microscope. Serological diagnosis (antigen detection) is available only in selected centres, and does not have high sensitivity (immunodiffusion - 58.8%, latex agglutination -76.5%). Antibody detection is not very helpful.

Safety and training

With regard to protective measures against blood exposure, the laboratory safety officer should ensure that all staff members, particularly new staff, receive adequate instruction. These instructions should ensure that the safety aspects of new practices and equipment have been considered and that standard practices are being followed. A laboratory safety manual should be maintained and updated at least annually. Supervisory staff must ensure that their staff adhere to the documented safe working practices. Staff should formally report needle stick injuries or other episodes of exposure to blood or body fluids.

Safety considerations

Specimen collections	<ul style="list-style-type: none"> • Universal precautions should be followed.
Specimen transport and Storage	<ul style="list-style-type: none"> • Sealed plastic bag (preferably double layered) with proper legible labeling.
Specimen processing	<ul style="list-style-type: none"> • Containment level II or III.
Specimen Disposal	<ul style="list-style-type: none"> • All infected material should be treated as per WHO procedures.

Specimen collection

Optimum time of specimen collection	<ul style="list-style-type: none"> • Ideally as close to the onset of symptoms as feasible. • Before initiation of antifungal therapy where possible. • In in-patients, early morning sample.
Correct specimen type	<ul style="list-style-type: none"> • Appropriate specimen according to the site of lesion, in adequate amount, collected with appropriate sterile implements and precautions.
Recommended amount*	<ul style="list-style-type: none"> • CSF 2-4 ml • Pleural fluid 10-20 ml • Bronchoalveolar lavage (BAL) 10 ml • Urine 10-50 ml • Urine (midstream) 10-20 ml • Sputum 2-5 ml <ul style="list-style-type: none"> – Coughed out sputum (not saliva) in a wide-mouthed sterile container; – In case of dry cough, induced sputum in sterile container; • Stool 1-5 g
	<ul style="list-style-type: none"> • Blood 5-10 ml <ul style="list-style-type: none"> – Inoculated directly to biphasic blood culture bottle remembering to maintain a ratio of 1:10 of blood to broth. – For separation of serum, additional 2-5 ml of blood to be put in clean, dry vials, preferably leak-proof screw-capped • Repeated sampling from the same site (for authentic diagnosis of opportunistic infection repeated demonstration/isolation of same organism from same site is essential)

* With larger quantity of specimen – yield increases.

Specimen transport and storage

Time between specimen collection and transport	<ul style="list-style-type: none"> Specimens should be transported to the specific laboratory as soon as possible.
Storage	<ul style="list-style-type: none"> Specimen should be processed in the laboratory as soon as possible. Delay in processing of un-refrigerated specimens over four hours is undesirable. Where there is a delay in processing, specimens should be refrigerated except CSF and specimens for isolation of <i>Cryptococcus</i>.

Specimen processing

Proper documentation upon receipt of specimen	<ul style="list-style-type: none"> Maintenance of laboratory records with lab number.
Initial processing	<ul style="list-style-type: none"> Depends upon the specimen concerned and common organism suspected Blood culture, straight incubation at 37°C. CSF and other body fluids, except blood, should be centrifuged in a separate sterile test tube or centrifuge tube, and supernatant poured back to the container of collection and stored for antigen detection. Centrifuge at 2 000 – 2 500 rpm for 10 minutes. Pellet is used for smear and wet mount, and subsequent culture.
Direct microscopy	<ul style="list-style-type: none"> Procedure employed depends upon specimen. Wet mount: <ul style="list-style-type: none"> KOH preparation India ink/Nigrosin staining Lacto Phenol Cotton Blue wherever applicable Stain/Smears <ul style="list-style-type: none"> Gram staining (for yeasts), Giemsa/ Wright staining (for <i>Histoplasma</i> and <i>Penicillium marneffeii</i>) Additional microscopic procedure: <ul style="list-style-type: none"> Direct fluorescence where applicable Direct and indirect immunofluorescence where applicable
Histopathology	<ul style="list-style-type: none"> Hematoxylin Eosin stain Periodic Acid Schiff stain Silver methanamine Mucicarmine stain
Culture	<ul style="list-style-type: none"> Given in a separate chart below.
Immunodiagnosis	<ul style="list-style-type: none"> Supernatant from centrifuged body fluids to be used for antigen detection wherever applicable

* Very important; can predict the nature of the infection.

Culture media and conditions

Type of specimen	Standard media	Incubation			Reading of cultures	Target organism	Comments
		Temp	Atmosphere	Time			
All specimen ¹ except blood	Specimen are inoculated in the following media: -Sabourau d's Dextrose Agar(SDA) -SDA + anti-bacterial antibiotic -SDA + anti-bacterial antibiotic and cycloheximide.	3 tubes 22-27°C	Aerobic	24-48h; some strains may take longer time	Daily; cultures to be incubated for at least 4 weeks before report negative	Filamentous forms of dimorphic fungi -Yeasts	
		3 tubes 35-37°C	Aerobic				
Blood culture ³	Biphasic brain heart infusion (BHI) Agar/broth biphasic medium with antibiotics for blood culture	37°C	Aerobic with vented bottle			Yeast, filamentous and yeast form of dimorphic fungi	This method of culture is cost-effective and gives uniformly satisfactory results with minimum risk of contamination and less load to the technical staff
Bone Marrow	Biphasic brain heart infusion (BHI) Agar medium with 5% sheep blood and antibiotics	37°C for yeast phase	Aerobic	48-96h		Dimorphic fungi ²	Prolonged incubation may be necessary

¹ Discussed under each infection

² Dimorphic fungi (e.g. *H. capsulatum*, *P. marneffei*)

³ Other available methods for blood culture:

Lysis centrifugation, Bactec, BacT Alert, etc.: These procedures are sensitive but costly, and require specific equipments and specially trained personnel.

Identification

Level	Specimen	Process	Reporting for positive findings, stating that if appropriate further report will be issued
Minimum level: 1 Direct microscopy	CSF, sputum, BAL, pus, mucus membrane, tissue biopsy, serum, scrapings, peripheral blood	KOH mount: presence of fungal filaments with characteristic branching and yeasts	Same day reporting: Fungal elements (branched septate hyphae, budding yeast, etc.) seen
		India Ink/Nigrosin for <i>Cryptococcus</i>	Same day reporting: Positive for encapsulated budding yeast compatible with <i>Cryptococcus</i>
		Gram staining: gram positive yeast cells with hyphae and pseudohyphae	Same day reporting: Yeast cells and septate hyphae seen, suggestive of <i>Candida</i> spp (organism)
		Giemsa/Wright stain: intracellular yeast with occasional budding	Same day reporting: Intracellular yeast cells seen suggestive of <i>Histoplasma</i>
		intracellular yeast, oval or elongated with transverse septae	Same day reporting: Intracellular yeast cells seen suggestive of <i>P. marneffe</i>
Serology (if applicable)	Serum, CSF, Urine	Antigen detection for presence of <i>C. neoformans</i>	Same day reporting: Sample positive for cryptococcal antigen (with/without titer)
Intermediate Level: 2 (Level 1 + Culture and serology)	Same as above	Identification depends on macroscopic and microscopic characteristics: <ul style="list-style-type: none"> - LCB mount for Filamentous fungi - Gram stain/ LCB mount for Yeast 	Same day reporting: _____ (organism) grown on culture
		Speciation: Germ tube test for presence of <i>C. albicans</i> <ul style="list-style-type: none"> - Positive: <i>Candida albicans</i> - Negative: Speciation is needed (assimilation, fermentation, and other biochemical tests) (Confirmation: by CMA) - Histopathology	Same day reporting: <i>C. albicans</i> was isolated Same day reporting: Yeast other than <i>Candida albicans</i> was isolated Same day reporting: Suggestive of fungal infection (organism)
Reference Level: 3 (Level 1 + Level 2)	1. All of above 2. Isolates for confirmation 3. External Quality control	<ul style="list-style-type: none"> • Strain differentiation • Antifungal susceptibility testing • Variety differentiation/ Serotyping of <i>C. neoformans</i> • Animal pathogenicity (if needed) • Histopathology 	Relevant report to be communicated to the person concerned as soon as possible.

Reporting should be done immediately upon a positive finding, and particularly in case of demonstration of organism in normally sterile body fluids, the clinician concerned should be informed immediately by phone, if possible.

Direct Examination

KOH mount preparation

- (1) Potassium hydroxide is a strong alkali used as a clearing agent to observe fungi in the specimen in a wet mount preparation.
- (2) 10-20% KOH is usually used depending on the specimen; occasionally 40% is also used.
- (3) It is used mainly to digest the keratin in the keratinized tissues present in the specimens, so that the fungal elements can be observed clearly.
- (4) Used ideally in suspected cases of dermatophytosis, *i.e.* fungal infection of skin, hair, or nails that contain keratin.
- (5) Also used for specimen such as sputum, pus, urine sediment, homogenate from biopsy tissue to clear cell debris.

Preparation of the mount

- (1) Take a clean grease-free glass slide.
- (2) Place a large drop of KOH solution with a Pastuer pipette.
- (3) Transfer small quantity of the specimen with a loop or the tip of a scalpel into the KOH drop.
- (4) Put a clean cover slip on gently so that no air bubble is trapped.
- (5) Place the slide in a moist chamber, and keep at room temperature.
- (6) Skin scales usually take 20-30 minutes; pieces of nail may take several hours to clear. Sometimes overnight contact with KOH is useful for getting a positive result.
- (7) Clearing can be hastened by gentle heating of the slide, but it is best avoided.

Observation

- (1) Examine the clear specimen under low power (10X objective). Scan the entire cover slip from end to end in a zig-zag fashion.
- (2) If any fungal elements are suspected, examine under high power (40X objective).
- (3) Reduce the light coming into the condenser.
- (4) Look for branching hyphae, type of branching, the colour, septation and thickness of hyphae, budding yeast cells.

Modification

- For more distinction, stains like methylene blue or Parker blue black fountain ink may be used along with KOH. This will impart a coloured background and fungal elements, if present, will show as prominent refractile objects.

Advantage of KOH preparation

- Simple, cheap and rapid

Disadvantage

- Pus and sputum may contain artefacts which may superficially resemble hyphal and budding forms of fungi. These artefacts may be produced by cotton or wool fibres, starch grains (in pleuritis) or cholesterol crystals.
- It gives an idea about the presence of hyphal element, but cannot distinguish different fungi.
- Preparation cannot be kept for too long.

Precautions

- (1) The droplet of KOH should not be so large that the coverslip floats.
- (2) If kept outside a moist chamber, the KOH dries and crystals form that restricts the visibility of the fungus.
- (3) After clearing, pressure is to be gently applied on the top of the coverslip with a fold of filter paper or the handle of a teasing needle. This ensures even spreading of the material onto the slide.
- (4) KOH should be kept in a closed container in small aliquots ready to use on the workbench.

Quality assurance

- Fungal spores may contaminate the KOH solution kept in the laboratory and may give false positive results. So a negative control should be put up every day.

India ink or Nigrosin preparation for identification of *C.neoformans*

- (1) The preparation is to be made in the centre of a clean, grease-free, glass slide.
- (2) Put one drop of India Ink or Nigrosin on the centre of the slide. Too much stain makes the background too dark. (Upon subsequent examination, if the staining appears too dark, a little amount of water may be applied on the edge of the cover slip and the coverslip gently tapped. This dilutes the stain to some extent).
- (3) Put one loopful of the specimen or preferably centrifuge sediment from the fluid specimen to be tested (e.g. CSF, spinal fluid, urine, other body fluids) close to the drop of the stain.
- (4) Mix the two well with the loop, or preferably a sterile needle. The loop should be cooled before use; otherwise, the stain tends to precipitate.
- (5) Hold the coverslip vertically such that one edge just touches the fluid on the slide. The fluid will spread on the edge by surface tension.

- (6) Keeping that edge in contact with the fluid surface, drop the coverslip gently on the fluid, so that no air bubble is trapped inside. If there are air bubbles, the surface of the coverslip may be gently tapped by the needle point, so as to move the bubbles towards the edge. but this should be avoided as far as practicable.
- (7) Examine slide immediately under the microscope. Since the stain tends to dry fast in air, if immediate examination is not feasible for the purpose of demonstration, the slide should be kept in a moist chamber (covered petri dish with a wet filter paper on which a triangular glass rod is placed).
- (8) Scan the entire cover slip from end to end in a zig-zag fashion. Encapsulated yeast *Cryptococcus neoformans* is seen under low power as luminous dots in an otherwise dark background. Under high power, the cells can also be seen, containing refractile nuclei, and surrounded by the unstained thickness of the capsule. Characteristic pinched-off budding, when observed, is confirmatory for diagnosis.

It should be noted specially that

- (a) Besides the classical budding-yeast form, various unusual forms can also be seen including elongated forms that look like pseudohyphae; this is mainly due to a very high multiplication rate of the organism in HIV/AIDS patients.
- (b) In the very late stage in progressive AIDS, it may be difficult to differentiate the capsules of individual cells; the organism may remain enmeshed in a matting of the capsular material.
- (c) Micro- or non-capsulated varieties of the organism are also reported on rare occasions. In such cases, Gram stain of the sample is helpful in identification.
- (d) The edges of the coverslip should be specially examined. While placing the coverslip, the yeast cells tend to move towards the periphery along with the fluid. For this reason the common practice of draining the extra fluid from the sides should be avoided.
- (e) If the protein content of the CSF is too high, India Ink sometimes may form floccules which make it difficult to demonstrate the capsule.
- (f) In case of Nigrosin stain, the preparation dries up quite fast which is a problem in hot climatic conditions. So quick examination is essential.
- (g) In case of HIV positive patients, $\geq 90\%$ of cases may be positive by the India Ink/Nigrosin test, whereas in non-HIV cases, $\leq 60\%$ positivity is seen.

Precautions

- (1) The India Ink or Nigrosin should be shaken well before every preparation.
- (2) The stain should be regularly checked for contamination by checking only the stain under microscope.
- (3) False positive readings may occur with air bubbles or monocytes. Air bubbles, under the high power, will be hollow and will not show the typical cell with characteristic nuclei. Monocytes have a crenated margin (and not the entire margin seen in cryptococcal cell), and will not show the characteristic nuclei, and the luminous halo around the cell is not well demarcated.

Lactophenol cotton blue (LCB) mount preparation

This is used for staining and microscopic identification of fungi.

Process

- (1) Take a clean grease-free glass slide.
- (2) Put a large drop of LCB with a Pastuer pipette.
- (3) Transfer a small quantity of the culture to the drop.
- (4) Tease the culture (in case of a mold) well with teasing needles so as to get a uniform spread.
- (5) Put on a coverslip gently to avoid entrapment of air bubbles.
- (6) Examine under low (10x) and high power (40x) of the microscope.
- (7) Observe the morphological features carefully.

Gram's staining

- (1) Make a very thin smear of the material on a clean grease-free glass slide.
- (2) Dry in air.
- (3) Fix the smear by flaming the slide.
- (4) Add gentian violet to cover the smear and leave undisturbed for 1 minute.
- (5) Drain off the gentian violet by tilting the slide and rinse in flowing tap water taking care that the water flow does not directly fall on the smear.
- (6) Add Gram iodine solution to cover the smear and leave for 1 minute.
- (7) Rinse with water in the same way as above.
- (8) Flood with acetone for about 30 seconds.
- (9) Rinse again with water.
- (10) Counterstain with safranin for 30 seconds, and rinse in water.
- (11) Dry in air, and observe.

Observation

- Gram reaction (positive or negative), size, shape and arrangement of elements should be observed under the oil immersion field.

Giemsa staining

- (1) Homogenize tissue section and make a thin uniform smear.
- (2) Flood the slide with methyl alcohol and leave for 3-5 minutes for fixation.
- (3) Add prepared Giemsa stain for 45 minutes.

- (4) Wash slide thoroughly with running tap water.
- (5) Blot dry with absorbent paper.
- (6) Observe under oil immersion lens.

Observation

- Intracellular budding yeast, as mentioned above.

Wright stain

- (1) Make a uniform smear (peripheral blood, bone marrow *etc.*) on a clean grease-free glass slide.
- (2) Cover the slide with freshly filtered Wright stain (it is important to cover the entire slide) and leave for 1-3 minutes.
- (3) Without removing the stain, pour on buffer solution (pH 6.4); surface tension will not allow the buffer to run off. Some workers prefer using tap water instead of the buffer solution.
- (4) Blow gently over the surface of the fluid to mix the buffer and the stain. Upon proper mixing a metallic green sheen (green scum) rises to the surface of the fluid. Leave for three minutes or more (*e.g.* bone marrow takes longer to stain).
- (5) Wash the slide gently with flowing tap water, and wipe the bottom of the slide with a clean tissue.
- (6) Air dry the slide, and observe under the microscope.

Precautions

- (1) Since Wright stain is prepared in methanol, a separate fixation step is not required.
- (2) The timings of each step should be standardized in the laboratory for optimal coloration.
- (3) The staining of different components in a smear is dependent on the pH of the medium, which has to be maintained. Excess alkaline or acidic conditions may cause the colour to be too blue or red to be seen properly.
- (4) Unfiltered Wright stain may leave granular particles on the preparation.

Observation

Intracellular budding yeast, as mentioned above; this is specially suitable for observation of intracellular *Histoplasma* and *P. marneffe* in bone marrow or peripheral blood smears.

Serology Test

Antigen testing for specific diagnosis of cryptococcosis

Cryptococcal antigen detection in body fluids is a reliable and very sensitive technique for authentic diagnosis of cryptococcosis. Commercial kits are available with excellent sensitivity and specificity.

- Of available methods of antigen detection, latex agglutination (LA) is simple, easy to perform, economical, and most popular.
- LA titer of $\geq 1:8$ is indicative of active infection. In case of HIV-positive patients with chronic meningitis, the titer in CSF may be extremely high, and antigen may also be detected in almost all body fluids. Antigen detection in urine may be a good non-invasive screening method.
- If antigen titer increases progressively, it indicates an unsatisfactory prognosis. Conversely, if with therapy it remains stationary or decreases, the prognosis is considered good, though in case of HIV-positive patients, it may take a long time to come down.
- In case of very high antigen concentration in the sample, the test may be falsely negative because of the prozone phenomenon. Therefore, in such a case, dilution of the sample to at least 1:100 before repeating the test is recommended.
- Occasional false positive reaction is also encountered, particularly in presence of rheumatoid factor or infection with the yeast *Trichosporon beigeli*. False positive reactions generally have a low titer ($\leq 1:4$). To reduce false positivity, pre-treatment of the sample with mercaptoethanol or pronase is recommended. Pronase treatment also helps dissolve the pre-formed antigen-antibody complexes in the sample.

Procedure

- Serum and CSF specimen for cryptococcal antigen testing must be heat inactivated at 56°C for a minimum of 30 minutes before being processed.
- Exact procedure to be followed should be according to the manufacturer's instructions.

Interpretation

- Agglutination pattern should be observed and interpreted according to the manufacturer's instructions.

Rapid identification germ tube test

Germ tube test is used for primary speciation of *Candida*. It is a rapid screening test where the production of germ tubes within two hours in contact with the serum is considered as indicative of *Candida albicans*. This test has to be confirmed with Corn Meal Agar (CMA) test.

Method

- (1) Ensure that the test starts with a fresh pure culture.
- (2) Make a very light suspension of the test organism in 0.5 ml of sterile serum. The optimum inoculum is 10^5 - 10^6 cells per ml.
- (3) Incubate at 37°C for exactly two hours.
- (4) Place one drop of the yeast serum on a slide with a coverslip. Observe under the microscope for production of germ tubes. Germ tubes appear as filaments that are not constricted at their point of origin on the parent cell.

- (5) To record a positive, about 30% of the cells should show germ tube production.
- (6) Suitable controls should be kept with each test; a known strain of *Candida albicans* should be tested with each new batch of serum.

Precautions

- (1) Increased concentration of inoculum causes a significant decrease in the percentage of cells forming germ tubes.
- (2) On prolonged incubation, non-*albicans Candida* spp may show germ tube production. For this reason incorporation of proper controls is important.

Germ tube negative yeasts isolated from a normally sterile body fluid or tissue should be identified to species level, for which the sample must be referred to the Reference Laboratory.

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Appendix

General techniques used in medical mycology

The techniques used in clinical mycology are in general similar to those used in clinical bacteriology. There are certain differences, however, which are important to note.

Basic equipment required

- (1) A stiff straight nichrome wire with a long handle – used for stabbing inoculation.
- (2) A bent (right angle) nichrome wire with long handle – used for handling the mycelial types of fungal cultures.
- (3) Bacteriological loop.
- (4) Pair of short stiff teasing needles - helpful in pulling apart dense masses of mycelium on the slide as a preliminary to microscopic examination.
- (5) Scalpel with blades.
- (6) A pair of scissors, for cutting biopsy tissues into small pieces.
- (7) Pair of forceps.

Test tubes should be used in preference to petri dishes for primary culture of organism from clinical specimen, or transferring fungal cultures. Test tubes afford the following advantages:

- a) Easy handling.
- b) Easy storage.
- c) Less frequent breakage.
- d) Greater protection to worker.
- e) Less drying of medium.

Cotton plugs in the test tubes for cultures are preferred over screw caps as they:

- (1) Allow the surface of the slants to remain dry.
- (2) Help better development of aerial mycelium.
- (3) Help sporulation.
- (4) Help in better pigment production.

Test tubes of fairly large diameter (18x150mm) are preferable to narrow tubes. These allow a thick butt of agar which will withstand drying during the several weeks often necessary for the growth of fungal cultures. When it is necessary to incubate cultures at 37°C, extra large tubes (25x150mm) are helpful.

At all times, for the protection of personnel and for securing and maintaining pure cultures, precise sterile techniques are to be employed.

It is suggested that a piece of absorbent paper such as paper towelling be placed beside the microscope, and that this be kept moist with some antiseptic solution. The Bunsen burner should be placed on, or at the edge of, this moistened paper. Containers of clinical material should be placed on this paper and culture tubes held over it when being examined.

20% KOH – glycerol Solution

KOH	20 gms
Glycerol ²	20 ml
Distilled water	80 ml

Note: 1. The relative amounts of KOH and distilled water have to be adjusted according to the percentage of KOH to be used.

2. Addition of glycerol to KOH solution will prevent crystallization occurring in the solution. Thus enhancing the shelf life of this reagent. It will also permit keeping the KOH preparation for a couple of days before it dries.

Lactophenol cotton blue (LCB)

LCB is used both as a mounting fluid and a stain. Lactic acid acts as a clearing agent and aids in preserving the fungal structures; phenol kills the organism and fixes it; glycerol prevents drying and cotton blue provides color to the structure. It can be used alone or in conjunction with KOH.

Lactic acid	20 ml
Phenol	20 ml
Glycerol	40 ml
Cotton blue (Poirrier blue or methyl blue)	0.05 g
Distilled water	20 ml

Phenol is to be dissolved in lactic acid, glycerol and distilled water; and cotton blue is then added and mixed well.

Corn Meal Agar (CMA)

CMA is used with Tween 80 in distinguishing the different species of *Candida* and can be useful in slide cultures as it stimulates formation of chlamydospores.

Cornmeal	40 g
Agar	20 g
Tween 80	10 ml
Distilled water	1000 ml

- (1) Mix cornmeal well with 500 ml of water.
- (2) Heat to 65°C for one hour.

- (3) Filter through a gauze piece and filter paper till clear solution is obtained.
- (4) Restore volume to 500 ml.
- (5) Adjust the pH to 6.6-6.8.
- (6) Add agar dissolved in 500 ml of water.
- (7) Add Tween 80.
- (8) Sterilize by autoclaving, and dispense into petri dishes or tubes.

For studying the morphology of yeasts, use one-fourth or one-third of a plate for each organism. Make one streak down the centre of the area (do not cut the agar) and three or four streaks across the first to dilute the inoculum. Cover with a 22 x 22-mm coverslip and incubate at room temperature for three days. Examine by placing the plate, without its lid, on the microscope stage and using the low-power (10x) and high-dry (43x) objectives. The most characteristic morphology is often found near the edge of the coverslip (especially the terminal chlamydoconidia of *Candida albicans*).

Candida albicans should always be included as a control for production of chlamydoconidia and blastoconidia.

Sabouraud's Dextrose Agar (SDA)

Emmons modification

This modification differs from the original formula in that it has an approximately neutral pH and contains only 2% dextrose.

Dextrose	20g
Peptone	10g
Agar	17g

To be dissolved in 1000 ml of distilled water.

The ingredients are to be mixed and dissolved by boiling and autoclaved. This formulation is available in prepared or dehydrated form. The final pH is around 5.6. The prepared medium can be stored at 4°C.

SDA with antibiotics

To SDA while boiling and before autoclaving add

Cycloheximide	500 mg dissolved in 10 ml of acetone
Chloaramphenicol**	50 mg dissolved in 10 ml of 95% ethanol

Wright stain

Wright stain is a Romanowsky type stain which contains methylene blue as the active ingredient. At the proper pH, the methyl groups are activated and react with charged components of the cell to produce a coloration.

* The antibiotic may be decided according to local conditions and bacterial strains prevalent.

Powdered Wright's stain	9 g (0.3% w/v)
Powdered Giemsa stain	1 g (0.033% w/v)
Glycerin	90 ml (3% v/v)
Absolute acetone-free methanol	2910 ml (to make up the volume)

The components have to be mixed in a brown bottle and let stand for one month before use. The stain must be stored at 4°C; otherwise the components may degrade.

6. Standard Operating Procedures for the Laboratory Diagnosis of Common Parasitic Opportunistic Infections in HIV/AIDS Patients

Introduction

Opportunistic parasitic infections can cause severe morbidity and mortality. Since many of these infections are treatable, an early and accurate diagnosis is important. This can be accomplished by a variety of methods such as direct demonstration of the parasite and by serological tests to detect antigen and/or specific antibodies. Adherence to conventional procedure may not be appropriate in patients with AIDS. For example, antibody response may be poor in these patients and therefore immunodiagnostic tests have to be interpreted with caution.

Pneumocystis carinii, *Cryptosporidium*, *Toxoplasma gondii*, *Microsporidia* and *Strongyloides stercoralis* are commonly detected parasites. Less common parasites are *Acanthamoeba*, *Cyclospora* and *Isospora belli*. Detection of these parasites will help in proper management and treatment of these patients since drugs are available for most of these opportunistic infections.

Emphasis will be made on the importance of these parasites and the methods of their diagnosis in HIV/AIDS patients.

Clinical association and diagnosis

Pneumocystis carinii, a ubiquitous extracellular protozoan is a common pulmonary pathogen in immunocompromised hosts and is one of the AIDS defining events as part of the CDC's case definition of AIDS. In such subjects, the disease manifests as interstitial pneumonia. Laboratory diagnosis is made by demonstrating cysts or trophozoites by Giemsa, Toluidine blue or Methenamine silver nitrate stain in lung biopsy, hypopharyngeal washings, transbronchial aspirations or transthoracic needle aspiration. Circulating antigen demonstration by ELISA or PCR is also confirmatory. For these tests, the samples will have to be sent to the reference laboratory.

Cryptosporidiosis, a disease commonly caused by *Cryptosporidium parvum* causes severe chronic and even fatal diarrhoea with malabsorption and dehydration. Diarrhoea is watery without blood and mucus, may be scanty or continuous as much as 12-17 litres per day. Extraintestinal infections in liver and lungs are known in AIDS patients. Laboratory diagnosis is made by demonstrating oocysts in the specimens using modified acid fast stain or safranin methylene blue technique.

Toxoplasmosis, a disease caused by an ubiquitous intracellular protozoan *Toxoplasma gondii*, is a world wide zoonosis. The cat is the definitive host for the sexual stage and produces oocysts that are eliminated in the stool. All other infected animals and humans are intermediate hosts. In an immunocompromised host the disease presents as toxoplasmic encephalitis. Diagnosis is made by CT scan showing characteristic cerebral hypodense lesions with ring-enhancement after IV contrast. For definitive diagnosis, brain biopsy is done to demonstrate the organism. Toxoplasma antigen demonstration by ELISA or PCR from blood or CSF is possible for which facilities may be available in reference laboratories or centres of excellence and samples need to be sent to these laboratories.

Microsporidiosis, a disease commonly caused by *Enterocytozoon bienneusii* and *Septata intestinalis*, causes intractable diarrhoea and weight loss in AIDS patients. Diagnosis is made by demonstrating *Microsporidia* spores in stool specimens by modified Trichrome stain or Gram Chromotrop stain and histologically from the intestinal biopsy.

Strongyloides stercoralis, an intestinal nematode, also known as dwarf tapeworm, is potentially lethal because of its potential to cause an overwhelming autoinfection in immunocompromised hosts. Diarrhoea is the major clinical manifestation. Eosinophilia and local or generalized rash may also occur. Definitive diagnosis is made by demonstrating larvae in faeces or duodenal fluid.

Cyclospora, another intestinal coccidian parasite causes prolonged, often relapsing watery diarrhoea and weight loss. Modified safranin staining technique is recommended to demonstrate oocysts in the faecal smear.

Isosporiasis caused by *Isospora belli* produces protracted and sometimes profuse diarrhoea. The oocysts are demonstrated in faecal smears by modified acid fast stain.

Safety considerations

Specimen collection	<ul style="list-style-type: none">• Universal precautions to be followed, avoid contact with specimens with ungloved hands.
Specimen transport and storage	<ul style="list-style-type: none">• Sterile leak-proof container in a sealed plastic bag.
Specimen processing	<ul style="list-style-type: none">• At least biosafety level II with good laboratory practice.

The above guidelines should be supplemented with recommendations from the local health hazard and risk assessment committee.

Specimen collection

Optimum time of specimen collection	<ul style="list-style-type: none"> • Ideally as close to the onset of symptoms as possible; • Before initiation of antiparasitic therapy; • First morning sample
Correct specimen type	<ul style="list-style-type: none"> • Waxed cardboard box with an overlapping lid, or a plastic cup or box with a tight-fitting lid and two applicator sticks • Three consecutive stool specimens at 1-day interval is ideal • For <i>P. carinii</i>, collect transbronchial aspirate, 10 ml bronchial washing or biopsy in plain sterile vial • For antigen detection collect 3-5 ml blood in plain vial or 1-2 ml CSF in a plain sterile vial

Specimen transport and storage

Time between specimen collection and processing	<ul style="list-style-type: none"> • The specimen must reach the laboratory within half an hour after passage. If this is not possible, specimens must be treated with preservatives like 10% formaline or PVA
Storage	<ul style="list-style-type: none"> • Specimen should be stored in ice box or refrigerator.

Specimen processing

Proper documentation upon receipt of specimen	<ul style="list-style-type: none"> • Maintenance of laboratory records with lab number. Laboratory request form should indicate the specific test for <i>P. carinii</i> and <i>T. gondii</i> • Laboratory record and laboratory number
Initial processing	<ul style="list-style-type: none"> • Depends upon the clinical criteria and parasite suspected • Stool sample; examine for consistency, blood, mucous or any adult parasite; • Blood sample; centrifuge at 1000-1500 rpm for five minutes and separate serum and store at -20°C for antigen detection; • CSF and other body fluids to be centrifuged at 2000 – 2500 rpm for 10 minutes, and supernatant collected and stored at -20°C for antigen detection; • Sediment to be used for smear and wet mount; • Blood specimen for PCR and antigen detection to be sent to the reference laboratory, and • Impression smear or necropsy or biopsy specimen for <i>P. carinii</i> and <i>T. gondii</i>.

Microscopy	<ul style="list-style-type: none"> • Very important; can predict the nature of the infection • Procedure employed depends upon specimen • Examine saline or iodine wet mount for protozoa and larvae, directly from faecal material or concentrated specimen • Giemsa stain or toluidine blue stain for <i>P. carinii</i> and <i>T. gondii</i> • Modified acid fast stain or Safranin methylene blue for <i>Cryptosporidium</i> and <i>Cyclospora</i> • Gram chromotrope or modified trichrome stain for <i>Microspodia</i>
Histopathology	<ul style="list-style-type: none"> • Hematoxylin eosin stain, PAS stain and methenamine silver nitrate stain for <i>P. carinii</i> • Hematoxylin eosin stain and PAS stain for <i>T. gondii</i>
Serology	<ul style="list-style-type: none"> • Supernatant from centrifuged blood or body fluids to be used for antigen or antibody detection where applicable.
Reporting procedure	<ul style="list-style-type: none"> • Reporting should include appearance, consistency, presence of blood, mucus, pus, worms or worm fragments • The report should mention the presence of RBCs, WBCs and parasite detected. The results should be reported immediately by telephone personally if possible.
Preservation of specimens	<ul style="list-style-type: none"> • Preserve stool samples in equal amount of 10% buffered formalin. • Screw the cap of the vial securely. • Wrap a piece of adhesive tape around the top of vial to prevent leakage. • Pack the vial(s) carefully in the box for sending to reference laboratory.
Disposal of specimen	<ul style="list-style-type: none"> • Stool samples collected in paper boxes, dispose of by burning the entire container. If collected in metal or glass container, add enough 10% formalin to kill the parasite. • Used slides to be put in a pan of disinfectant such as 1% sodium hypochlorite solution for at least one hour before washing. • Push the coverslip off the slide into the disinfectant pan with applicator stick. • All used glassware to be put into disinfectant pan for at least one hour before washing. • Applicator sticks and gauge pieces should be burnt.

Collection of faecal specimens

Because of the fragile nature of many intestinal parasites, and the need to maintain their morphology for accurate identification, reliable microscopic diagnosis cannot be made unless the stool is collected properly.

Give the patient the following:

- A waxed cardboard box with an overlapping lid, or a plastic cup or box with a tight-fitting lid
- Two applicator sticks

If waxed boxes or plastic cups are not available, tin boxes or glass jars can be used. Three specimens are usually recommended, at one-day intervals, to detect all parasitic infections. A variety of substances may interfere with the examination of stool specimens for parasites (*e.g.* laxatives, antacids, ingested contrast media and certain antibiotics).

The container with the specimens should be labelled clearly with the following information:

- Patient's name or number
- Date of collection
- Time the patient passed the stool (ask the patient when he/she passed the stool).

Tell the patient to pass the stool specimen directly into the container, or to pass the stool on to a piece of paper and use the applicator sticks to transfer it to the container. If paper is not available, the faeces can be passed on to a large, clean leaf. However, the stool must be transferred immediately to the specimen container. It should not remain on the leaf or be brought to the laboratory on the paper/leaf.

The stool specimen must be large enough for satisfactory examination. The smallest quantity that should be accepted is about the size of a pigeon's egg. Urine and dirt should be excluded. Urine will destroy any protozoan trophozoites and dirt will interfere with the examination. If the specimen is too small, or if it is mixed with urine or dirt, it should not be accepted. Ask the patient to pass another specimen.

Specimen transport and storage

- (1) Some organisms, especially amoebic trophozoites, will begin to disintegrate or change within a short time after passage and become unrecognizable. Warm temperatures will hasten these changes. Therefore, specimens must reach the laboratory very soon (*i.e.*, within half an hour) after passage. If this is not possible, the specimen must be treated with preservatives.
- (2) Keep the carton containing the specimen in a refrigerator, or if this is not possible in the coolest, shadiest area in the laboratory. Do not keep the specimen artificially warm and do not leave it in the sun.

Specimen examination

Macroscopic examination of stool

- (1) As soon as the specimen is received in the laboratory, check the consistency (degree of moisture) and write one of the following on the container: formed, soft, loose, or watery. The consistency, or degree of moisture, will be a guide as to whether the trophozoite stage is in the stool. The appropriate techniques to be used are shown in the Table.
- (2) If several specimens are received at the same time, those containing blood and mucus should be examined first followed by liquid specimens. These specimens are the most likely to contain amoebic trophozoites (which die soon after being passed) and must be examined within an hour after passage. Formed specimens may be examined at any time during the first day, but should not be left overnight (cysts may disintegrate).

Microscopic examination of Wet Mounts

Wet mounting is the simple and easiest technique for examination of faeces, and this method should be performed in all laboratories at the peripheral level.

A wet mount can be prepared directly from faecal material or from concentrated specimens. The basic types of wet mount to be used for each faecal examination are saline and iodine.

The *saline wet mount* is used for the initial microscopic examination of stools. It is employed primarily to demonstrate worm eggs, larvae, protozoan trophozoites, and cysts. This type of mount can also reveal the presence of red blood cells and white blood cells.

Categories of Stool and Appropriate Techniques to be used

Consistency	Protozoan stage most likely to be found*	Technique to be used		
		Saline	Iodine	Buffered Methylene blue
Formed	Cysts	+	+	+
Soft	Cysts	+	+	+
	(occasionally trophozoites)			+
Loose	Trophozoites	+		+

*Worm eggs and larvae may be found in stools of any consistency

- The Iodine wet mount is used mainly to stain glycogen and the nuclei of cysts, if present. Cysts can usually be specifically identified in this mount.
- Buffered Methylene Blue (BMB) wet mount should be prepared every time amoebic trophozoites are seen in a saline wet mount or when their presence is suspected. BMB stain does not stain amoebic cysts. It is only appropriate for fresh unpreserved specimens.

Staining Methods

Direct saline, Iodine Mounts and BMB Mounts

Materials and reagents

- (1) Coverslips
- (2) Dropping-bottles containing: saline solution (isotonic), Lugol's iodine (1% solution), 1% BMB stain
- (3) Microscope slides
- (4) Pens or markers for labeling
- (5) Wire loop (or applicator sticks, matchsticks, or toothpicks).

Technique

- (1) With a wax pencil write the patient's name or number and the date at the left-hand end of the slide.
- (2) Place a drop of saline in the centre of the left half of the slide and place a drop of iodine solution in the right half of the slide.

Note:

If the presence of amoebic trophozoites is suspected, warm saline (37°C) should be used. In case of BMB stain, wait for five minutes to allow the stain to penetrate the trophozoites. It will overstain the trophozoites in 30 minutes. Therefore the slide must be examined as early as possible.

- (3) With an applicator stick (match or toothpick), pick up a small portion of the specimen (size of a match head) and mix with the drop of saline.

Note

Formed stool: Take the portion of stool from an area to include inside and outside parts of the specimen.

Stool with mucus: If mucus is present, label a second slide with the patient's name or number. Put a drop of saline on the slide, pick up a small portion of mucus and mix with the saline. Trophozoites, if present, are sometimes more readily found in mucus than in the solid parts of the stool.

Loose watery stool: If mucus is not present, pick up a small portion of the stool (any part) and mix with the saline.

- (4) Similarly, pick up a small amount of the stool and mix with the drop of iodine, to prepare an iodine mount. If a wire loop is used, flame it after making the mount. If applicator sticks are used, discard them.
- (5) Cover the drop of saline and the drop of iodine with a coverslip. Hold the coverslip at an angle, touch the edge of the drop and lower gently on to the slide. This will reduce the chance of including air bubbles in the mount.

Concentration techniques

Formalin ether concentration technique

If the number of organisms in the stool specimen is low, examination of a direct wet mount may not detect parasites. Thus, whenever possible, the stool should be concentrated. Worm eggs, larvae and protozoan cysts may be recovered by concentration but protozoan trophozoites will NOT be seen as they are usually destroyed during the concentration procedure. This makes direct wet mount examination obligatory as the initial phase of microscopic examination.

The concentration procedure is indicated when the initial wet mount examination is negative despite the clinical symptoms indicating parasitic infection of a patient, and for the detection of *Schistosoma* and *Taenia*.

The concentration procedure recommended is the formalin-ether (or formalin-ethyl acetate) method. All types of worm eggs (roundworms, tapeworms, schistosomes, and other fluke eggs), larvae, and protozoan cysts may be recovered by this method.

Materials and reagents

- (1) Applicator sticks, wooden
- (2) Bottles, dispensing or plastic "squeeze", 250 ml or 500 ml. These bottles are convenient for adding formalin to the centrifuge tubes. However, any small bottles or flasks may be used.
- (3) Centrifuge, with head and cups to hold 15-ml conical tubes. Sealed buckets must be used.
- (4) Centrifuge tubes, 15 ml, conical (make a graduation at 7 ml and 10 ml with a grease pencil).
- (5) Cotton swabs
- (6) Coverslips
- (7) Funnel
- (8) Surgical gauze
- (9) Microscope slides
- (10) Pipettes, Pasteur, with rubber bulbs
- (11) Rack or support for tubes
- (12) 10% Formalin. For everyday use, pour some of the solution into a "squeeze" bottle. Label the bottle.
- (13) Ether or ethyl acetate.
- (14) Lugol's iodine, 1% solution - in a dispensing bottle with a pipette
- (15) Saline solution, isotonic

Caution

Ether is a highly flammable compound and will ignite and explode quickly if there is a flame or spark nearby. Store opened cans or bottles on an open shelf in the coolest part of the laboratory. Be sure the cans or bottles are stoppered. DO NOT put an opened container of ether in a refrigerator: fumes build up inside the refrigerator, even if the container is closed and may explode when the door is opened. DO NOT put opened containers in a cabinet. It is better to leave the container on an open shelf so that the fumes can disperse readily.

Technique

- (1) Add 10 ml of 10% formalin to approximately 1 g of faeces and stir an applicator stick, until you get a slightly cloudy suspension. Fit a gauze filter into a funnel and place the funnel on top of the centrifuge tube.
- (2) Pass the faecal suspension through the filter into the centrifuge tube until the 7 ml mark is reached.
- (3) Remove the filter and discard the filter with the lumpy residue.
- (4) Add 3 ml of ether or ethyl acetate and mix well for one minute.
- (5) Transfer back to the centrifuge tube and centrifuge for 1 minute.
- (6) Loosen the fatty plug (debris) with an applicator stick, and pour away the supernatant by quickly inverting the tube.
- (7) Replace the tube in its rack and allow the fluid on the slides of the tube to drain down to the sediment. Mix well and transfer a drop to a slide for examination under a coverslip. Also make an iodine-stained preparation.
- (8) Use the X 10 and X 40 objectives to examine the whole area under the coverslip for ova, cysts, and larvae.

Concentration technique for cryptosporidium oocysts

Flotation or sedimentation is particularly helpful in recovering oocysts from non-liquid stool specimens. Oocysts float easily in Sheather's sucrose solution, in zinc sulphate (33% to saturated), and in sodium chloride (36% to saturated). They pellet with formalin ethyl acetate or formalin ether.

Permanent staining techniques

Permanently stained slides are not made routinely in diagnostic practice and are not required for the identification of worm, eggs or larvae. However, permanently stained preparations are occasionally required for the following purposes:

- Identification of oocysts of *Cryptosporidium*;
- Identification of protozoan trophozoites or cysts, if doubt exists;

- Confirmation of the identified protozoan cysts, where doubt exists;
- Keeping a permanent record, and
- Sending to a reference laboratory for an expert opinion.

Staining Technique for Oocysts of *Cryptosporidium parvum*

Oocysts of *Cryptosporidium* passed in faeces are spherical, measuring 4-6µm in diameter. They may be concentrated using a modified formalin-ether technique, but must be identified by staining methods. The recommended method is the modified Ziel - Neelsen technique.

Modified Ziehl - Neelsen Technique

Materials

- (1) Applicator sticks, wooden
- (2) Coverslips
- (3) Forceps
- (4) Microscope slides
- (5) Pen or marker for labeling
- (6) Rod, glass
- (7) Slide holder, for finished slides
- (8) Small bottle of mounting medium
- (9) Staining dishes
- (10) Paper towel or sponge

Reagents

- (1) Carbol – fuchsin, filter before use
- (2) Formalin (formaldehyde)
- (3) Hydrochloric acid - ethanol solution
- (4) Glycerol - malachite green (or methylene blue) solution
- (5) Hydrochloric acid - methanol solution
- (6) Water

Preparation

- (1) Make a thin faecal smear, leave it to air-dry and fix it with a flame for a few seconds or in methanol for 2-3 minutes. Further fixation in formalin vapour should be performed if possible, to reduce infectivity. (The sediment from formalin - ether extraction cannot be used.)
- (2) Flood the smear with cold carbol-fuchsin.
- (3) Heat the slide until steaming but do not allow drying.

- (4) Allow to stand for approximately 5 minutes.
- (5) Rinse slide in tap water and drain.
- (6) Decolourize with 5% H₂SO₄ or 1% HCl-ethanol until color ceases and flood out (1-2 minutes).
- (7) Rinse slide in tap water and drain.
- (8) Counterstain with methylene blue or 0.25% malachite green for 1-2 minutes.
- (9) Rinse in tap water.
- (10) Blot or drain dry.
- (11) Examine using first x10 and then the high-power, dry objective x40 and confirm the morphology using oil immersion. Measure the cysts. *Cryptosporidium* cysts measure 4-6 µm and appear as bright rose-pink spherules on a pale green background.

Gram-chromotrope stain for Microsporidia

Materials and Reagents

- (1) Microscope slides
- (2) Coverslips
- (3) Applicators sticks, wooden and glass
- (4) Forceps
- (5) Pen or marker for labeling
- (6) Slide holder, for finished slides
- (7) Small bottle of mounting medium
- (8) Seven staining dishes
- (9) Paper towel or sponge (if not available newspaper)
- (10) 1% Crystal violet
- (11) Gram's iodine solution
- (12) Ethyl ether acetone (equal concentrations of ethyl ether and acetone)
- (13) Chromotrope stain

Chromotrope 2R	6.0 g
Fast green	0.15 g
Phosphotungstic acid	0.7 g
Glacial acetic acid	3.0 g
Distilled water	100 ml
0.45% acid alcohol	

Preparation of chromotrope stain

Weigh each dye powder separately. Put the dyes into a 100-ml flask. Weigh out phosphotungstic acid crystals and add to the flask with the dyes. Measure the glacial acetic acid and pour into the flask. Swirl the flask so that the acetic acid wets the dyes. Let it stand for 30 minutes. Then mix with 100 ml of distilled water and adjust the pH to 2.5 with 1 M HCl. Pour the stain into a 100-ml, clean, glass-stoppered bottle. Label the bottle

as Gram Chromotrope stain and write the date. Store on a shelf or in cabinet away from the light. The stain will remain good for a year or more.

Preparation

- (1) Make a faecal smear and let it dry at room temperature.
- (2) Heat fix (Three times for 1 sec. each over a low flame).
- (3) Flood the slide with Crystal violet solution.
- (4) Rinse with tap water.
- (5) Flood slide with Gram's iodine and allow to remain on the slide for 1 min.
- (6) Rinse gently with tap water (1-2 sec).
- (7) Flood with tap water.
- (8) Place slide in Chromotrope stain for at least 5-10 min.
- (9) Rinse in 0.45% acid alcohol for 1-3 sec.
- (10) Rinse in 95% ethanol for 1 min.
- (11) Rinse in absolute ethanol for 30 sec twice.
- (12) Let it dry at room temperature.

Examination

- (1) Put the slide with the mounts on the microscope stage and focus on the mount with the 10x or low-power objective.
- (2) Regulate the light in the microscope field with the substage diaphragm. You should be able to see objects in the field distinctly. Too much or too little light is not good.
- (3) Examine the entire coverslip area with the X 10 objective: focus the objective on the top left-hand corner and move the slide systematically backwards and forwards, or up and down.
- (4) Use the 40x objective to identify small parasites.
- (5) Use the oil-immersion objectives to examine protozoan and coccidia.

This is a systematic examination. If mounts are examined in this way, any parasites present will usually be found. If the mount is not examined systematically, parasites may be missed. Examine each microscopic field carefully, focusing up and down, before moving to the next field.

Toluidine Stain for *Pneumocystis carinii*

Staining Procedure

- (1) Dry slide for 5 minutes.
- (2) Flood slide with sulphonation reagent for 10 minutes.
- (3) Note: Wear gloves and take care when handling this reagent.

- (4) Carefully wash off reagent and flush down sink.
- (5) Place slide in a container and flush with running water for 5 minutes.
- (6) Flood slide with Toluidine blue for 3 minutes.
- (7) Wash off and allow slide to air dry.
- (8) Examine using 10x objective where clumps of cysts may be visible and 40x and under oil immersion for confirmation.

Results

Pneumocystis carinii cysts of characteristic morphology and size 3-5 µm are violet or purple against a bluish background.

Sulphonation reagent

Fresh reagent is made once a week. To 9 ml of glacial acetic acid in a bottle held in a cool water bath (10-15°C), slowly add 3 ml of concentrated sulphuric acid. Mix and leave in the water bath until the solution cools.

Toluidine blue

Dissolve 0.3 g of Toluidine blue in 60 ml of distilled water. Add 2 ml of concentrated hydrochloric acid followed by 140 ml of absolute ethyl alcohol.

Remember: Examine mounts systematically

Identification of parasites

Worm eggs and larvae in saline mounts

Eggs may be easily detected and identified in saline mounts. They should not be stained (stains may interfere with identification). Most of the eggs are large enough to be recognized with the low-power 10x objective, but a few small eggs will require a high-power dry lens. In saline mounts, larvae of *Strongyloides stercoralis* may be seen. Hookworm larvae are not usually present if the sample is fresh, but it may be necessary to distinguish between these two species if an old sample is examined.

Protozoa in wet mounts

Saline wet mounts

In saline mounts, trophozoites and cysts of amoebae (cysts of *Isospora belli*) and flagellates may be seen. Cysts will appear as round or oval, refractile structures; the trophozoites of amoebae may be round or irregular; the trophozoites of flagellates are usually pyriform (elongated, pear-shaped). In freshly passed faeces (the stool must not be more than an hour old), motile trophozoites may be seen. Motility can be very helpful in identifying species, especially in the case of flagellates.

Organisms may be detected with the low-power 10x objective, but a high-power, dry objective will be necessary to reliably identify the structure as a cyst or trophozoite. With the high-power, dry objective, you can see motility, inclusions like erythrocytes and yeast in amoebic trophozoites, chromatoid bodies in amoebic cysts, and the shape and structural details (*e.g.* sucking discs, spiral grooves, or filaments) of flagellate trophozoites and cysts. Oocysts of *Isospora belli* are ellipsoidal, 22-33 x 12-15 μm size, containing two sporocysts each. You will not be able to see any detail in the nucleus in saline mounts. However, it is necessary to regulate the microscope illumination carefully so that the objects appear clearly. Too much or too little light will interfere with your observations. It is also necessary to focus up and down to see all the layers (levels) of the specimen. Remember to examine the whole coverslip area in a systematic manner to reduce the chances of overlooking organisms.

BMB mount

The nucleus and inclusions will stain dark blue and the cytoplasm will stain light blue. Look for peripheral nuclear granules around the nucleus for *Entamoeba* species.

Iodine wet mount

Iodine mounts are examined for protozoa and coccidian cysts. They can be detected with the 10x objective, but they are not as refractile as in saline mounts. High-power dry magnification must be used to see the characteristics of the cysts and they must be measured to ensure correct identification. In the iodine mount, cytoplasm of the cysts will stain yellow or light brown and nuclei will stain dark brown.

In iodine-stained cysts of *Entamoeba*, the arrangement of the peripheral chromatin and the position of the karyosome can be seen. (If the peripheral chromatin is not present, the cyst is not *Entamoeba* species.) These peripheral chromatoid bodies stain light yellow and may not be very clear. Sometimes, young cysts contain glycogen; this stains dark brown with iodine.

In iodine-stained flagellate cysts, the fibrils (filaments) can be seen.

In iodine-stained coccidian cysts, a central undivided mass of protoplasm can be seen.

Specific identification of amoebic and flagellate cysts can usually be made from iodine wet mounts. However, occasionally a definite identification cannot be made, and it may be necessary to use permanent stains.

Modified Ziehl - Neelsen Stain

When stained by Ziehl -Neelsen technique, *Cryptosporidium* oocysts appear as bright rose-pink spherules generally 4-6 μm in diameter, in a pale green background. The color may be unevenly distributed due to variable carbol fuchsin uptake by the oocyst wall, especially in rapidly shed young with a less mature wall.

Yeast cells and faecal debris assume the color of the blue or green counter stain. Although sometimes sporozoites can be clearly seen within the oocysts, size is important in

differentiating oocysts from other organisms with similar staining properties. *Cyclospora* spp. resemble *Cryptosporidium parvum* but are roughly twice the size, i.e. 8-10 µm in diameter. As this is unsporulated, they show a spherical cytoplasmic mass.

The large oocysts of *Isospora belli* contain two sporocysts measuring 12-14 x 7-9 µm each with four sporozoites of 20-23 x 11-19 µm in size elongated or ellipsoidal in shape with no residuum (in freshly passed stool, only one sporocyst may be seen).

Modified gram chromotrope stain for *Microsporidia*

When stained by Gram chromotrope staining technique, *Microsporidia* spores are ovoid and refractile. They measure approximately 0.9-1.5 µm in diameter. The spore wall stains bright pinkish red. The cellular content of some spores appears transparent and some spores show a distinct pinkish-red-stained belt-like stripe that girds the spores diagonally or equatorially. Although other faecal elements, such as yeast, sometimes stain reddish, they can be distinguished from *Microsporidia* spores by their large size and more intense staining. Most bacteria and background debris counterstain faint green.

Reporting procedure

Reporting should include appearance of specimen:

- Consistency
- Blood, mucus, pus
- Worms or worm fragments

The written report should include WBCs, erythrocytes, and organisms detected. The written report should be submitted within 24-72 hours. In urgent cases the result should be reported immediately by telephone (if available) or personally.

Preservation of specimens

Materials and reagents

- (1) Adhesive tape
- (2) Applicator sticks, wooden
- (3) Bottles, 1000 ml
- (4) Labels
- (5) Pen or marker for labeling
- (6) Vials, 20 ml, with tight-fitting screw-caps
- (7) 10% formalin (formaldehyde)

Technique

- (1) Label two 20-ml vials with patient's name or number. Write F in the upper right-hand corner of the label on one vial.
- (2) Fill the "F" vial about half full with 10% formalin.

- (3) With an applicator stick, pick up a portion of the stool to include areas from the inside and edges of the sample and mix with the 10% formalin. Be sure to mix very well; break up lumps. Use enough, but not too much stool so that the mixture will occupy about 2/3 to 3/4 of the vial.
- (4) Screw the caps of the vials securely. Wrap a piece of adhesive tape around the top of each vial to prevent leaking.
- (5) Pack the vials carefully in a box or shipping container and send to the reference laboratory. Be sure that the vials are surrounded by absorbent materials (e.g. cotton wool, newspaper) and are packed so they will not break.
- (6) Be sure to include the necessary information: patient's name or number, date of shipping, organisms you found.

For long-term preservation, faecal samples may be stored in 2.5% (weight/volume) potassium dichromate in which oocysts remain for as long as six months.

Disposal of specimens

- (1) If stools are collected in paper boxes, the way to dispose of them is by burning the entire container. If they cannot be burnt, or if the stool was collected in a metal or glass container, add enough 10% formalin to cover the stool left in the container. This will kill any parasites that might be present. Allow to stand for an hour or more before discarding or washing (if the container is glass).
- (2) Slides used for wet mounts should be put in a pan of disinfectant (e.g., sodium hypochlorite) for at least an hour before washing. Use an applicator stick to push the coverslip off into a breaker or small pan of disinfectant and then put the slide into another pan of disinfectant.

Coverslips break easily, and if put in with the slides, they may break and cut the hands of the person washing them.

- (3) Funnels, stoppers, and centrifuge tubes should also be put into disinfectant for an hour before washing.
- (4) Applicator sticks and gauze squares should be burned. If burning is not possible, they can be discarded after soaking in disinfectant.

Quality assurance for faecal examination

To ensure accurate and reliable results, good laboratory practices must be applied to laboratory procedure for diagnosing parasitic infections. Quality assurance must apply to collection of specimens, preparation of reagents, performance of the techniques, examination of the preparations and reporting. National level laboratories may be motivated to organize National External Quality Assessment Schemes for strengthening the quality assurance programme especially Internal Quality Control for the regional/peripheral laboratories.

Diagnostic procedures to be performed at different levels

Level 1: Peripheral level

- Smear examination for intestinal protozoa and Helminths
- Smear examination for *P. carinii*
- Smear examination for *Cryptosporidia*

Level 2: Intermediate level (regional hospital, provincial hospital /university)

Histopathology for *P. carinii*, *T. gondii*

Antigen demonstration

Special stains for Microsporidia, Cryptosporidia,

Cyclospora, Isospora, *P. carinii* and *T. gondii*

Level 3: Central level (reference laboratories and centres of excellence)

Demonstration of antigen by ELISA, PCR and probes

Strain differentiation

References

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- (3) Forbes BA, Sahm DF, Weissfield AS Diagnostic Microbiology, 1998, 10th edition.
- (4) Moura H et al. Gram-chromotrope (1996): A new technique that enhances detection of *microsporidial* spores in clinical sample. J Eukaryot Microbiol; 43 (5): 94S-95S.

Appendices

Reagents and Solutions

Lugol's iodine (Stock 5% Solution)

Iodine	5 g
Potassium iodide (KI)	10 g
Distilled water	up to 100 ml

Dissolve the potassium iodide in about 30 ml of the water. Add the iodine and mix until dissolved. Add a further 70 ml of water and mix well. Store in a brown bottle.

Lugol's iodine (1% Solution for wet mounts)

Lugol's iodine (stock, 5% solution)	5 ml
Saline solution, isotonic	20 ml

Measure the isotonic saline into a dispensing or dropping bottle. Add the 5% Lugol's iodine stock solution. Mix thoroughly. This will give a 1% iodine solution which will satisfactorily stain cysts.

Buffered methylene blue stain

Methylene blue powder	1 g
Disodium hydrogen phosphate	3 g
Potassium dihydrogen phosphate	1 g
Distilled water	300 ml

Weigh out the methylene blue powder and put in a clean dry mortar. Add the disodium hydrogen phosphate and potassium dihydrogen phosphate. With a pestle, grind the dye and phosphate powders together and mix thoroughly. Weigh 1 g portions of the mixture and put in a small well-stoppered vials.

Preparations of stain

Put 1 g of the mixture in a 500 ml flask. Add the distilled water and shake the flask or stir to dissolve the dye mixture. Filter through filter paper into a 500 ml clean, dry, glass-stoppered bottle. This stain will remain good for two years or more.

PVA-fixative preparation

Glycerol	1.5 ml
Polyvinyl alcohol (PVA) powder (low viscosity)	5.0 g
Distilled water	62.5 ml

In a small beaker, add the glycerol to the PVA powder and mix thoroughly with a glass rod until all particles appear coated with the glycerol. Scrape the mixture into a 125 ml flask. Add the distilled water, stopper, and leave at room temperature for three hours or overnight. Swirl mixture occasionally to mix.

Carbol-fuchsin solution

Basic fuchsin	10 g
Ethanol, absolute, technical grade	100 ml
Phenol	50 g
Distilled water	1 l

Preparation

Weigh the basic fuchsin powder and transfer it into a 1.5 litre bottle. Add 100 ml of absolute ethanol and dissolve the dye completely. Weigh the phenol in a beaker and dissolve in a small volume of distilled water. Add the aqueous phenol solution to the dye solution and mix well. Add the rest of the water, mix well and label the bottle. The dye solution will be stable indefinitely.

Hydrochloric acid-ethanol solution

Hydrochloric acid (concentrated)	1 ml
Ethanol 95%	100 ml

Put 100 ml of 95% ethanol into a clean 250 ml bottle with glass stopper. Add 1 ml of concentrated hydrochloric acid and then mix.

Glycerol-malachite green solution (or methylene blue) solution

Glycerol	100 ml
3% Aqueous malachite green, or 3% aqueous methylene blue	1 ml
Distilled water	100 ml

Grind some malachite green or methylene blue powder with a pestle in a clean, dry mortar. Weigh out 3 g of the powder, pour it into a bottle and add distilled water to give 100 ml. Seal and label the bottle 3% aqueous malachite green or 3% methylene blue. Store in the cabinet away from light.

To prepare the solution, add 1 ml of 3% aqueous solution into a 250 ml bottle. Add 100 ml of glycerol and 100 ml of distilled water and seal the bottle and mix thoroughly before use.

Hydrochloric acid-methanol solution

Hydrochloric acid (concentrated)	3 ml
Methanol, absolute	100 ml

Measure 100 ml of absolute methanol and pour into a clean 250 ml bottle with glass stopper. Add 3 ml of concentrated acid and mix.

Annex

LIST OF PARTICIPANTS WHO LABORATORY CONSULTATIVE MEETING ON STANDARD OPERATING PROCEDURES FOR DIAGNOSIS OF HIV-OPPORTUNISTIC INFECTIONS, 27-30 SEPTEMBER 1999

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