# THE CLINICAL MICROBIOLOGY LABORATORY IN THE DIAGNOSIS AND MONITORING OF OPPORTUNISTIC INFECTIONS IN HIV-INFECTED PERSONS

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## Abstract

The patient with AIDS is at risk for infection with a myriad of organisms spanning the full spectrum from viruses to parasites. In many instances, etiologies associated with the more compromised AIDS patient rarely if ever cause disease in the immunologically stable host, thus possibly making it difficult to recognize the pathogens and to interpret their role in disease. Newer methods of testing, including direct antigenic/genetic material detection by enzyme immunoassays, monoclonal fluorescent studies or genetic probes, as well as by molecular amplification systems are on the brink of revolutionizing the routine approach to diagnostics. Unfortunately, the increased sensitivity of the test methods often compromises their specificity, further increasing the difficulty in result interpretation.

Notwithstanding the problems inherent in test methodologies, more etiologies than ever have been associated with infections in the immunocompromised host. In most instances, newer methods can detect fungi (such as *Pneumocystis carinii*, *Cryptococcus neoformans*, *Histoplasma capsulatum*, and *Coccidioides immitis*), parasites (such as cryptosporidia, microsporidia, and cyclospora), and mycobacteria (such as *Mycobacterium tuberculosis*, *Mycobacterium avium* complex, *Mycobacterium genavense*, and *Mycobacterium haemophilum*) quite readily. It is important that carefully formulated testing pathways or formats be utilized to allow for timely and correct evaluation and diagnosis, thus allowing for institution of appropriate therapy and, hopefully, good outcomes.

## Introduction

Depending on the extent of debilitation in the immune system, an HIV-infected person is prone to invasion by and increased pathology due to an extremely wide array of microorganisms. As the CD4 counts decrease, due to continued HIV replication, the list of potential pathogens continues to increase, spanning the full spectrum from viruses to parasites. At the lower limit of immunity, even microorganisms normally considered non-invasive or commensal become invasive pathogens with capability to cause not only morbidity but also mortality. With the introduction and acceptance of antiretroviral agents in the treatment of patients with HIV disease, the incidence of AIDS-defining illnesses has declined substantially. However, their overall spectrum remains unchanged. Thus, diagnostic techniques in the laboratory must include means by which to detect these vast and very disparate groups of organisms. In most instances, techniques need to include various microscopic methods and cultures incorporating a variety of media and incubation environments.

Serologic studies are usually suboptimal for the timely diagnosis of disease in the immunologically compromised host, although they may play a contributory role only in such instances as the diagnosis of coccidioidomycosis (Valley Fever) and toxoplasmosis. Methods to detect invasive organisms by enzyme immunoassay, fluorescent tags or other tagging technologies also play roles, but may be inconsistent in their sensitivities, specificities and positive as well as negative predictive values.

Molecular amplification techniques have only recently been introduced into the clinical laboratory and are now being evaluated for their routine application in patient care. Unfortunately, such methods are most frequently not standardized at the present time, thereby potentially providing results from different laboratories having poor agreement, thus creating confusion as to their interpretation and use in the clinical setting. Additionally, the increased sensitivity of these methods often compromises their specificity, further increasing the difficulty in result interpretation. Standardization of methods and evaluation of outcome studies are critical if amplification techniques are to become useful and cost-effective in the diagnosis and management of infectious disease in HIV-infected patients.

Because many pathogens found in the immunologically compromised host may also be found in the environment or as colonizers, it is frequently essential to confirm an isolate's pathogenic role through histologic evaluation of the site of infection. This is especially true if the organism is detected at a non-sterile site such as sputum or skin lesion. A close working relationship and facilitated communication between the microbiology and histologic sections of the laboratory should be encouraged and supported.

## *Laboratory Overview*

### Upper Respiratory Tract

Evaluation of patients with HIV disease presenting with oral lesions should include brushings and/or biopsy for histologic analysis and culture for viruses and fungi. Of the fungi, the yeast Candida is most frequently associated with such lesions, although Cryptococcus, Histoplasma, and Geotrichum may occasionally be involved. Viral etiologies usually include Herpes simplex virus (HSV), Cytomegalovirus (CMV), Epstein-Barr virus (EBV), Varicella-Zoster virus (VZ), and Papilloma virus (PV) which is the causative agent of oral hairy leukoplasia.

Etiologic agents of esophageal disease in the HIV seropositive patient include an expanded spectrum of microorganisms. Specimens for evaluation should include esophageal brushings and endoscopically collected biopsies. *Candida albicans* and *Candida glabrata* can be detected by the Gram or calcofluor white stains (a fluorescent stain preferentially binding to the chitin in fungal cell walls) or the histologic Periodic-Acid Schiff (PAS) and/or Grocott-Methenamine Silver (GMS) stains, or by culture. Far less commonly, Histoplasma may be found in esophageal lesions after hematogenous dissemination; fungi such as Aspergillus, the zygomycetes, and Cryptococcus may be detected rarely either by histologic stain or culture. On occasion, esophageal lesions may be caused by such mycobacteria as *M. tuberculosis* and *M. avium* complex which may be detected by acid fast stain or culture. Of the viral etiologies, CMV is most common and may be implicated by histologic staining for characteristic intranuclear inclusions. HSV and EBV may also be found and these are best detected by in-situ hybridization studies.

### Lower Respiratory Tract

The overall spectrum of etiologies and incidence rates of lower respiratory tract infections in HIV-infected patients are shown in Table 1. The most common causes are shown in Table 2. Bacterial infections are still the most common and the case definition of AIDS was revised in 1993 to include recurrent bacterial infection. Association between bacterial groups causing progressive disease and cellular defects include: a) suppression of T cell function with Legionella, Nocardia and *Rhodococcus equi*; b) dysfunction of B cells with the pyogenic bacteria, especially *Streptococcus pneumoniae* and *Haemophilus influenzae*; and c) abnormal neutrophil function in chemotaxis, phagocytosis and intracellular

killing with *Staphylococcus aureus* and the Gram- negative bacilli.

Evaluation of AIDS patients with pulmonary infiltrates should include the collection of expectorated or induced sputum, bronchoscopically collected material, blood, and possibly CSF and urine for a variety of stains, cultures and antigenic determinations as clinically indicated (Table 3). One should remember that infection rates with certain organisms may vary geographically depending on specific endemic areas (e.g. histoplasmosis, coccidioidomycosis, nocardiosis, and legionellosis) and consideration should be given to residence area and/or travel history. Geographic variation may also occur in prevalence of specific antigenic serogroups of organisms and thus increase or decrease sensitivities and specificities of many studies. As an example, *Legionella pneumophila* serogroup 1 seems to be the prevalent cause of legionellosis in Northeast United States, but serogroups 4, 6, and 8 or even other species of Legionella may predominate in the Southwest. Thus, diagnosis of legionellosis by detection of serogroup 1 antigen (which is the only serogroup for which a test is currently available) in urine is compromised in the Southwest where sensitivity of such studies drops drastically.

## Gastrointestinal Tract

One of the most difficult infectious processes to find an etiologic agent for is diarrhea, with comprehensive exams yielding 50-80 % of the causes. In North America and Europe 30-60 % of patients with AIDS develop diarrhea, while in Africa the number approaches 90 % . Of the bacteria, Campylobacter spp., Clostridium, enteropathogenic *E. coli*, Salmonella, Shigella and other common causes of diarrhea in the immunocompetent host also remain primary etiologies in the HIV-infected person.

Detection of the various bacteria requires several different selective media and incubation atmospheres and temperatures. Additionally, diarrhea in this set of patients can also be caused by acid-fast bacilli (AFB) in the *Mycobacterium avium* complex (MAC), viruses such as herpes simplex (HSV), cytomegalovirus (CMV), and adenovirus, and an ever increasing number of parasitic etiologies such as Giardia, *Entamoeba histolytica*, Isospora, Cryptosporidia, Microsporidia, and Cyclospora. Full scale evaluations become very time-consuming and expensive in the laboratory; thus, clinical parameters should guide the clinician in determining the extent of the studies to pursue. Studies may include routine bacterial cultures of stool specimens (to detect Campylobacter, Shigella, and Salmonella), other bacterial cultures as indicated (e.g. *E. coli* O157 which may not be detected by routine culture), assay for *Clostridium difficile* toxin when indicated (culture for the organism is not normally helpful), culture and acid-fast staining for mycobacteria, stool viral studies by electron-microscopy (for Corona-like viruses, etc.), or enzyme linked immunoassay (ELISA; for adenovirus, astrovirus, rotavirus), and parasitic examinations. Parasitic exams should include not only the normal stains for traditional parasites, but also the newly introduced and more sensitive direct fluorescent methods or ELISA for Cryptosporidia and Giardia. Diagnosis of microsporidia is best accomplished by using the Warthin-Starry silver stain or the Brown and Brenn gram stain for histologic evaluations of tissue specimens; fluorochrome stains such as calcolfluor white and the modified trichrome stains should be used on fluid specimens such as urine, stool and mucus. Modified acid-fast stains and/or fluorescent calcofluor white stains are helpful in detection of Cryptosporidia, Cyclospora and Isospora. When evaluating for bacterial or parasitic etiologies, studies on 2-3 stool specimens over 6-10 days may increase yields significantly. Biopsies collected endoscopically or by other means may be necessary in cases of recalcitrant or long term diarrheas.

## Diagnosis of Bacterial Infections

Most commonly, diagnosis of bacterial infection in the laboratory presently follows fairly traditional methods of staining and culture. Interpretation of the significance of isolates depends on isolate identity, site of infection, and method of specimen collection. Isolates from deep-seated, normally sterile sites are usually considered as being more significant than those from sites easily colonized or contaminated by organisms frequently found in the environment. Sputum specimens should still be evaluated for presence of epithelial versus white blood cells and for the presence of an identifiable organism or organisms associated with the white cells; contaminated specimens should not be worked up in most circumstances for routine bacterial culture. Careful microscopic evaluation should also be performed on all specimens from non-sterile sites for possible contamination (presence of epithelial cells, absence of white cells, presence of multiple or singular species of organisms representative of environmental or colonizing flora) and workup of such contaminated specimens should be avoided.

### *Diagnosis of Mycobacterial and Mycotic Infections*

The lists of mycobacterial and fungal species causing systemic disease in the HIV-infected patient continue to grow. The common mycobacterioses and mycoses are listed in Tables 4 and 6, respectively.

## Mycobacterioses Update

Improvements in laboratory isolation, as well as in identification and susceptibility testing of the mycobacteria, has decreased the time to the diagnosis of both traditional tuberculosis caused by *Mycobacterium tuberculosis* (Mtb) and infections caused by the nontuberculous mycobacteria (NTM).

Newer technology has decreased the average time to recovery of acid fast organisms in culture from 21 to 9 days; the combined recovery and identification of Mtb and MAC can now be accomplished in 10-12 days instead of the 4 to 6 weeks of old. Furthermore, susceptibility studies of Mtb against primary antituberculous agents isoniazid, rifampin, streptomycin and ethambutol now take only an additional 5 days rather than 2-4 weeks from time to recovery. These changes have been made possible because of the introduction of new radiometric broth techniques for isolation and susceptibility testing, and of molecular probe techniques for identification of Mtb, MAC, and *M. kansasii*. The introduction of high-pressure liquid chromatographic methods to identify the mycobacteria by evaluation of the characteristic presence of long carbon chain fatty acids (mycolic acids or mycolates) in the cell wall has allowed for the rapid and accurate identification of the NTM as well as of Mtb. The recently described method (polymerase chain reaction (PCR)/restriction enzyme analysis or PRA) consisting of PCR amplification of a 439-base pair segment of a heat shock protein –65 gene found in aerobic actinomycetes , followed by restriction enzyme analysis, has been successfully applied to the identification of over 50 species of aerobic actinomycetes. Although it can identify species of Mycobacteria, Nocardia, Rhodococcus, Actinomadura, Streptomyces, and Tsukamurella, it still lacks defined data for identification of the slower growing Mycobacteria and the pigmented rapidly growing species of Mycobacteria.

Other, molecular amplification methods have become available and FDA approved for the diagnosis of Mtb in smear positive sputum specimens. Such studies have high sensitivity and specificity (>95%) when smears show AFB, but poorer predictive values with microscopically negative specimens where sensitivity drops to approximately 60%. Their clinical utility depends therefore on the probability or heightened clinical suspicion for TB with each individ-

ual case. Although amplification techniques are not yet available for the direct diagnosis of NTM in specimens, the recent introduction of fluorescent columns for use in HPLC analysis, as well as the potential application of PRA, may make direct studies possible in the near future.

Unlike Mtb, the NTM are not transmitted person-to-person but rather are acquired from the environment where they are commonly ubiquitous. Reservoirs may include natural as well as tap waters (MAC, *M. kansasii*, *M. marinum*, many others), soil (rapidly growing mycobacteria, *M. malmoense*), or even dogs and pet birds (*M. genavense*). In some instances the environmental source eludes us (*M. haemophilum*, *M. szulgai*). Because the NTM are so common, they may easily be isolated from clinical specimens as transient contaminants or colonizers. Thus, interpretation of their presence in cultures or smears must be made carefully. Diagnostic criteria have been proposed by the American Thoracic Society (ATS) and should be followed diligently.

Although rapid and traditional susceptibility tests whose results correlate with clinical outcome are available for Mtb, routine susceptibility testing of the NTM is discouraged and often can provide erroneous and misleading information. Some recommendations have been made by the ATS for the susceptibility testing of the NTM and are summarized in Table 5.

## Mycoses Update

As can be recognized in Table 6, certain mycoses with geographic variation are causes of morbidity in patients residing or visiting specific areas. Thus, histoplasmosis (most common), blastomycosis (least common) and coccidioidomycosis play large roles in their respective endemic areas (histoplasmosis and blastomycosis: Mississippi and Ohio River Valleys in the U.S. and in Latin America; coccioidomycosis: Southwestern U.S. and parts of Latin America). Other fungi such as *Cryptococcus neoformans* and other hyaline moulds are found fairly universally and are not as geographically restricted. Moulds more recently recognized as increasing in importance as causes of severe, systemic acute or chronic disease include *Sporothrix schenkii*, some members of the dematiaceous fungi (darkly pigmented; including the genera Bipolaris, Xylohypha, Alternaria, Curvularia, *Pseudallescheria boydii* and its asexual synanamorph *Scedosporium apiospermum*, and *Scedosporium prolificans*) and *Penicillium marneffei*. The last is emerging as an important HIV-related pathogen in Southeast Asia and is the only dimorphic species in the genus Penicillium, whose members have been traditionally saprophytic in nature and have been contaminants in the laboratory Almost any fungus is now known to be capable of causing disease in the patient with extensive enough immunosuppression, although histopathologic documentation of involvement is commonly necessary to confirm the diagnosis. Laboratory diagnostic methods include smears, cultures and in a few instances serologies.

*Pneumocystis carinii* has been determined to be more closely related to the fungi than to the parasites and is now classified with this group of organisms. The organism cannot be cultured, but diagnosis has been expedited through the introduction of monoclonal fluorescent stains, whose sensitivities when used on cytospun induced or bronchoscopically collected lower respiratory secretions approaches 69-92% and surpasses the sensitivities of other stains such as giemsa, toluidine O, or GMS.

In most instances, however, culture on fungal media and identification of a fungal isolate either by determination of characteristic phenotypic morphology (moulds) or in combination with biochemical studies (yeasts) is still necessary to make a diagnosis. Genetic probes are now available for the rapid identification of *Coccidioides immitis*, *Histoplasma capsulatum*, and *Blastomyces dermatitidis*; these probes allow very

accurate identification within a few days of isolation of the pathogens circumventing the need to perform immunodiffusion precipitin studies to confirm identities even without characteristic structures or coversion studies converting the isolates from mould to yeast phase at ambient temperatures.

Serologic techniques are not normally helpful for the clinically relevant diagnosis of most fungal infections except in a few notable cases. Serologic studies for *Coccidioides immitis* precipitins (IgM) and IgG antibodies by either an enzyme immunoassay, an agar-gel diffusion or by complement fixation (CF) methods (CF looking for IgG) are very helpful not only in the diagnosis but also in the evaluation of the activity of disease (prognosis). The latter can be followed by increasing CF titers which are directly related to increases in disease activity. Detection of cryptococcal antigen in cerebrospinal fluid (CSF) and serum has high sensitivity and is useful for both diagnosis of cryptococcosis and monitoring its response to therapy by watching dropping titers over a course of time. Radioimmunoassay for detection of a heat stable polysaccharide in serum or urine of patients with histoplasomosis has a high degree of sensitivity and specificity (>90%) in the diagnosis of disseminated disease (available through Dr. Joseph Wheat's laboratory at the Indiana University School of Medicine).

A recent clinically relevant study showed that routine fungal blood cultures in patients with HIV disease are probably contraindicated. Fungal blood cultures in 322 patients with HIV yielded no clinically relevant isolates which had not already been recovered either through regular blood cultures or from other sites. On the other hand, fungal isolates were recovered in 11 fungus blood cultures which were determined to be contaminants; outcomes of routine culture in this setting could therefore be more confusing than diagnostically helpful.

Susceptibility studies are becoming more frequently necessary as resistant isolates accumulate and become more prevalent. Not only have azole-resistant *Candida albicans* isolates surfaced, but species of Candida intrinsically resistant to the azoles are becoming more commonly isolated as etiologies of disease. *Candida glabrata* may develop resistance to the azoles during therapy, but also display higher minimal inhibitory concentrations (MICs) against the azoles as a whole. *Candida krusei* has MICs considered resistant against the azoles. Well defined methods for in vitro susceptibility testing are becoming more widely available and a standardized broth dilution method has been recommended by the National Committee for Clinical Laboratory Standards. Further studies are needed to define more fully the clinical role of susceptibility studies and their correlations to patient outcomes under differing clinical circumstances.

## Molecular Techniques

A number of molecular techniques have been introduced for the detection or identification of microorganisms causing disease. A commercial emphasis has been placed on introduction of reagents for the high volume diseases such as urogenital infections due to chlamydia and the gonococcus. Other infectious processes have lagged behind in commercial development of specific reagents and molecular techniques, although methods for the detection of Mtb were recently approved by the FDA and are mentioned above. Other amplification or otherwise molecular systems include hepatitis C virus, HIV and Human Papilloma Virus (HPV). Noncommercial ("home brew") applications include PCR for HSV, enteroviruses, and other etiologies of disease, but their use is often limited to the institution in which they were set up and not broadly available; thus, with a lack of wide experience, their performance in the diagnosis of disease is still hard to define.

Still, the application of molecular techniques to clinical care situations is at present expanding and an area in which it is more highly advanced is in the diagnosis and viral load evaluations of CMV in various immunocompromised patients. CMV causes significant morbidity and mortality in the HIV-infected patient, affecting up to 40% of such patients during their course of illness. In most situations CD4 counts in symptomatic patients decrease below 100 mm3. The virus seeds the eyes, the gastrointestinal tract and, as recently recognized, the central nervous system.

Recent studies have shown a correlation between a high systemic CMV load and CMV disease. CMV load also seems to predict development of disease, response to therapy and disease prognosis, although a few patients with CMV retinitis have low or undetectable viral loads. Several methods have been used to show the association between viral load and disease progression, including CMV antigenemia assay, whole blood PCR, and plasma PCR, but no single method has been shown to be better than another at this time. Recent recommendations include monitoring patients with CD4 counts of less than 50 mm3 every 2 months for CMV load. Patients with plasma or whole cell CMV DNA of any level may require prophylactic therapy, although a higher threshold may apply for the more sensitive PMN-based antigenemia assay.

The role of quantitative viral load testing in prognosis and monitoring of response to therapy is just now being scrutinized. A major concern at this time is the lack of standardization of methods, so that results of various studies may not be directly comparable. Once such standardization occurs, evaluations of molecular techniques for both diagnosis and monitoring of the clinical cause of an infectious process will be simplified tremendously.

**Table 1. More common microorganisms causing HIV-associated pulmonary disorders**

**Bacterial:**
*S. pneumoniae*, *H. influenzae*, *S. aureus*, *M. catarrhalis*, *P. aeruginosa*, other Gram Negative Bacilli, including Legionella; Rhodococcus, Nocardia, Mycobacterium tuberculosis, Mycobacterium avium Complex, other Nontuberculous Mycobacteria

**Fungal:**
*Pneumocystis, Cryptococcus, Blastomyces, Histoplasma, Coccidioides, Aspergillus*

**Parasitic**:
Strongyloides , Toxoplasma, Cryptosporidium

**Viral:**
Herpes virus (CMV, HSV, VZV, EBV), Adenovirus, Influenza, RSV, Measles, Rhinovirus

*Adapted from Rosen

**Table 2. Incidence rates of lower respiratory tract infections in 1116 HIV seropositive patients.**

| Infection | # Patients | % |
|---|---|---|
| Bacterial pneumonia | 53 | 4.8 |
| Pneumocystis | 43 | 3.9 |
| NTM | 12 | 1.1 |
| Mtb | 10 | 0.9 |
| Nonspecific pneumonitis | 8 | 0.7 |
| Cryptococcus | 5 | 0.5 |
| CMV | 3 | 0.3 |
| Other (1 each Herpes, Toxo, Histo, lung abscess) | 1 each | 0.4 |

Adapted from Wallace

**Table 3. Evaluation of AIDS patients with pulmonary infiltrates**

| | |
|---|---|
| Sputum or Induced Sputum | Pneumocystis (DFA, Giemsa, GMS)<br>Usual bacterial pathogens (GS, culture)<br>Fungal culture (Crypto; Histo, Coccy)<br>AFB Culture and stain<br>Legionella (culture, DFA??) |
| Blood and/or CSF | Cryptococcus (India ink, latex) |
| Blood and/or urine | Histoplasma (EIA) |
| Urine | Legionella urinary antigen |

Adapted from Meduri

**Table 4. Major mycobacterial diseases in HIV-infected persons**

| | |
|---|---|
| M. tuberculosis | Effects all levels of immunity; pulmonary, extra-pulmonary with lowered immunity |
| *Mycobacterium avium* Complex | 50 CD4; disseminated; BCBs for diagnosis and treatment monitoring. |
| *M. kansasii* | <200 CD4; pulmonary (25% extrapulmonary); significant |
| *M. genavense* | <50 CD4; similar to MAC; difficult to grow |
| *M. xenopi* | Severe immunocompromised; disseminated; respiratory isolates may not be significant. |
| *M. haemophilum* | <200 CD4; cutaneous, septic arthrits,etc; requires heme, lower temperatures to grow. |
| *M. fortuitum*, chelonei, abscessus | Rapidly growing Nontuberculous Mycobacteria |

Adapted from Chin et. al

**Table 5. Recommendations for the *in vitro* susceptiblity testing of the nontuberculous mycobacteria.**

| NTM Species | Recommendation |
|---|---|
| *Mycobacterium avium* | Test clarithromycin only, and only if patient fails empirical prophylactic therapy *Mycobacterium kansasii*: Test rifampin only, and only if patient has been previously treated. |
| Rapidly growing Mycobacteria | Amikacin, doxycycline, imipenem, fluoroquinolones, sulfonamides, cefoxitin, clarithromycin...Base therapy on susceptibility profile. |
| Other NTM | Review individual needs and availabliity of in vitro study protocols (nonstandardized). |

Adapted from American Thoracic Society Guidelines, 1997.

**Table 6. Major Mycoses in AIDS**

| | |
|---|---|
| Candidiasis | >90% HIV worldwide; mucosal |
| Pneumocytosis | Usually pulmonary, rarely disseminated. |
| Cryptococcosis | % to 15% HIV |
| Histoplasmosis | 30% HIV locally; Midwest US/Latin America Disseminated infection/respiratory |
| Coccidioidomycosis | Locally common; Southwest US/Latin America Primary an reactivation; respiratory and disseminated |
| Blastomycosis | Rare/pulmonary disseminated;<br>Midwest US/scattered worldwide |
| Paracoccidioidomycosis | Rare/disseminated |
| Penicilliosis (marneffei) | Locally 20% HIV; Northern Thailand/China<br>Disseminated pulmonary/skin |
| Aspergillosis | Uncommon, but need to be considered |
| Zygomycosis | Uncommon, but need to be considered |

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