SECTION 1

Philosophy and Approach to Diagnostic Parasitology

Practical Guide to Diagnostic Parasitology, Third Edition. Lynne S. Garcia
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Neglected Tropical Diseases

The term "neglected tropical diseases" (NTDs) was first used in the early 2000s, primarily reflecting the lack of research funds and limited interest of the health care and pharmaceutical industries in investing in affordable drugs for these diseases. One of the earliest conferences on NTDs was organized by Médicins sans Frontières in early 2002 (1). From 2003 to 2007, key steps were taken to develop a framework for tackling NTDs in a coordinated and integrated way (2). In 2005 and 2006, two important articles (3, 4) provided a list of 15 NTDs, 13 of which were deemed of particular importance in terms of annual mortality rates and global burden. This list of 15 diseases formed the initial scope of *PLoS Neglected Tropical Diseases*. Included were nine helminth infections (cysticercosis/taeniasis, drancunculiasis [guinea worm], echinococcosis [added by WHO], foodborne trematodiasis, lymphatic filariasis, onchocerciasis, schistosomiasis, the three main soil-transmitted helminthiases [ascariasis, hookworm infection, and trichuriasis]), three protozoal infections (Chagas' disease, human African trypanosomiasis, and leishmaniasis), scabies and other ectoparasite infections (added by WHO), and three bacterial infections (Buruli ulcer, leprosy, and trachoma) (Table 1.1) (2).

A subsequent review by Hotez and colleagues titled "Control of Neglected Tropical Diseases," published in the *New England Journal of Medicine* in 2007, clearly demonstrated that the term "neglected tropical diseases" had become mainstream (5). In October 2007, the Public Library of Sciences published the inaugural issue of a new open-access journal, *PLoS Neglected Tropical Diseases* (6). As of mid-November 2020, more than 8,200 original research papers, editorials, expert opinions, viewpoints, and other magazine-type articles on NTDs have been published.

In a publication from 2017, Dr. Hotez discussed the tremendous progress towards neglected tropical disease control or even elimination. However, there are important gaps, nine of which are discussed below; parasitic infections have been emphasized for this list (7).

The first group of problems is linked to the geopolitics of the NTDs.

1. **Regional significance**. There are several NTDs that are very important in the areas where they occur; however, they are generally ignored by the global community. Examples include loiasis in Central Africa and mucocutaneous leishmaniasis in the New World.
2. **Political unrest in the Old World.** Second only to poverty, conflict may have the largest social impact on NTDs. Both cutaneous and visceral leishmaniasis outbreaks are now arising in Syria, Iraq, Afghanistan, Sudan, and South Sudan. Cutaneous leishmaniasis has now reached hyperendemic proportions in current and former ISIS occupation zones, and through forced human emigrations, this NTD may spill over into Lebanon, Turkey, and Jordan.
3. **Political destabilization in the New World.** As Venezuela's health system continues to decline, we have seen the resurgence or reemergence of malaria and NTDs such as Chagas' disease and schistosomiasis.
4. **Climate change and its impact on vector-borne and zoonotic NTDs.** Climate change, along with poverty, war, and population movements, produces detrimental effects which include the increase and spread of NTDs.
5. **NTDs in "wealthy" nations.** The poor living in the Group of 20 (G20) nations—and also Nigeria (richer than the bottom three or four G20 nations)—account for a majority of the world's disease burden for poverty-related neglected diseases and NTDs. These numbers include millions of Americans living in the United States with an NTD and significant but often unrecognized levels of poverty and disease in Europe and Australia.

The second group is related to coverage gaps and providing universal access to treatment.

6. **Female genital schistosomiasis.** Female genital schistosomiasis is one of the most common gynecologic conditions of women who live in poverty in Africa and is one of Africa's most important cofactors in its AIDS epidemic.
7. **Patient access to essential drugs for Chagas' disease**. Today, most cases of Chagas' disease occur in Latin America's three large economies: Argentina, Brazil, and Mexico. However, more than 90% of people with *Trypanosoma cruzi* infection do not have access to treatment.
8. **Mass drug administration (preventive chemotherapy).** This includes drugs against scabies, lymphatic filariasis, onchocerciasis, and schistosomiasis.
9. **Research and development (R&D)** for a single approach rather than multiple new approaches for control and disease elimination. For malaria, we will need to pursue several R&D approaches, including new drugs, diagnostics, vaccines, and vector control approaches.

A subsequent article by Dr. Hotez and colleagues in 2018 anticipated a number of challenges related to the emergence and reemergence of these diseases (8). These challenges include stress from climate change and catastrophic weather events, regional conflicts over shifting and limited resources, such as water, and the development and spread of urban helminth infections (schistosomiasis and toxocariasis), foodborne trematode infections, cysticercosis, protozoan infections, and zoonotic toxoplasmosis.

Why Perform Diagnostic Parasitology Testing?

Travel

With the increase in world travel and access to varied populations and geographic areas, we continue to see more "tropical" diseases and infections outside areas of endemicity due to the rapidity with which people and organisms can be transmitted from one place to another. Travel has also become accessible and more affordable for many people throughout the world, including those whose overall health status is in some way compromised. The increased transportation of infectious agents and potential human carriers, particularly via air travel, has been clearly demonstrated during the last few years. It has also been well documented that vectors carrying parasitic organisms can be transported via air travel in baggage and in the unpressurized parts of the plane itself; once released, these infected vectors can then transmit these parasites to humans, even in areas where the infections are not endemic.

Population Movements

In many parts of the world, particularly where conflict is ongoing, there continue to be large population movements. Such movements include refugee migrations to and from areas of endemic parasitic diseases (9, 10). Often, in refugee situations, living conditions are very poor and medical limitations may lead to high levels of parasitic disease and severe illness. Also, migrants may move into countries and geographic areas where serious parasitic infections are generally nonendemic, including Europe and parts of North America. Even if these individuals are uninfected when entering these areas, travel home to visit relatives may result in infections that can be imported when they return.

Control Issues

Control of parasites that cause disease is linked to a number of factors, including geographic location, public health infrastructure, political stability, available funding, social and behavioral customs and beliefs, trained laboratory personnel, health care support teams, environmental constraints, degree of understanding of organism life cycles, and opportunities for control and overall commitment. Often control efforts do not cross political or geographic boundaries; unfortunately, vectors and other carriers of infectious agents do not "play by the rules," and as a result, these boundaries are meaningless in the context of disease control.

Climate Change

With the continued increase in the global temperature, worldwide climate changes are leading to an overall increase in infectious diseases, vector populations, and ranges of endemicity of both parasites and vectors. Global warming enhances the potential spread of tropical parasitic infections, specifically those due to parasites such as *Plasmodium* spp., *Leishmania* spp., and *Trypanosoma* spp. (11). Examples of vectors whose range is increasing include *Anopheles, Aedes*, and *Culex* mosquitoes, hard ticks, and triatomid bugs. Another example is the vectors of schistosomiasis (12).

Epidemiologic Considerations

When newer infectious agents and/or diseases are recognized, there is often very little information available regarding the organism life cycle, potential reservoir hosts, and environmental requirements for survival. Priorities may change, and epidemiologic considerations may have been moved lower on the priority list in areas of the world where they were considered important in the past; unfortunately, funding often plays a role in decisions that impact disease control measures.

Compromised Patients; Potential Sex Bias Regarding Infection Susceptibility; Aging

With the tremendous increase in the number of patients whose immune systems are compromised by underlying illness, chemotherapy, transplantation, AIDS, or age, we are much more likely to see increasing numbers of opportunistic infections, including those caused by parasites. Also, we continue to discover and document organisms that were thought to be nonpathogenic but can cause serious disease in the compromised host. When the possible cause of illness in this patient population is being assessed, the possibility of parasitic infections must be considered as part of the differential diagnosis.

Various studies have revealed a bias toward males regarding susceptibility to and severity of parasitic diseases. Although a number of external factors influence the exposure to infection sources among males and females, one recurrent factor suggests that hormonal influence impacts the simultaneous increase in disease occurrence and hormonal activity during the aging process. However, to date, very few controlled studies have been performed. Hormones are suspected to play a role in parasitic disease processes such as amebiasis, malaria, leishmaniasis, toxoplasmosis, and schistosomiasis (13).

There are various examples of the relationship of parasite pathogenesis and the aging population. Parasite biomass, endothelial activation, and microvascular dysfunction are associated with severe disease in *Plasmodium knowlesi* malaria and likely contribute to pathogenesis. The association of each of these processes with aging may

account for the greater severity of malaria observed in older adults in regions of low endemicity (14).

Approach to Therapy

As new etiologic agents are discovered and the need for new therapeutics increases, more sensitive and specific diagnostic methods to assess the efficacy of newer drugs and alternative therapies will become mandatory. Skilled laboratorians, physicians, public health personnel, and other health care team members will be required to think globally in terms of infectious diseases caused by bacterial, fungal, parasitic, and viral agents, particularly when certain parasitic infections require very specific therapeutic regimens.

Who Should Perform Diagnostic Parasitology Testing?

Laboratory Personnel

Diagnostic procedures in the field of medical parasitology require a great deal of judgmental and interpretative experience and are, with very few exceptions, classified by the Clinical Laboratory Improvement Act of 1988 (CLIA '88) as high-complexity procedures. Currently, very few procedures can be automated for routine laboratory use, and organism identification relies on morphologic characteristics that can be difficult to differentiate. Although morphology can be "learned" at the microscope, knowledge about the life cycle, epidemiology, infectivity, geographic range, clinical symptoms, range of illness, disease presentation depending on immune status, and recommended therapy is critical to the operation of any laboratory providing diagnostic services in medical parasitology. As laboratories continue to downsize and reduce staff, cross-training will become more common and critical to financial success. Maintaining expertise in fields such as diagnostic parasitology has become more difficult, particularly when standard manual methods are used. Also, the lower the positive rate for parasitic infections, the more likely it is that the laboratory will generate both false-positive and false-negative laboratory reports. It is important for members of the health care team to thoroughly recognize areas of the clinical laboratory that require experienced personnel and why various procedures are recommended above others.

Nonlaboratory Personnel

Health care delivery settings where physicians provide parasitology diagnostic testing occasionally provide "simple" test results (CLIA '88 waived tests) based on wet-mount examinations. However, in spite of the CLIA classification of these diagnostic methods, wet-mount examinations are often very difficult to perform, and results are often incomplete or incorrect. Currently, there are no specific "over-the-counter" testing methods for parasitic infections; however, the future may see some newer diagnostic developments in this area. The key to performance of diagnostic medical parasitology procedures is formal training and experience. As the laboratory setting continues to change, it is important to recognize that these changes will require a thorough understanding of the skills required to perform diagnostic parasitology procedures and the pros and cons of available diagnostic methods. **Laboratories will have a number of diagnostic options; whatever approach is selected by an individual laboratory, the clinical relevance of the approach must be thoroughly understood and conveyed to the client user of the laboratory services.**

Where Should Diagnostic Parasitology Testing Be Performed?

Inpatient Setting

Most diagnostic parasitology procedures can be performed either within the hospital setting or at an offsite location. There are very few procedures within this discipline that must be performed and reported on a stat basis. **Two procedures fall into the stat category: request for examination of blood films for the diagnosis of malaria or other blood parasites and examination of cerebrospinal fluid (CSF) for the presence of free-living amebae, primarily *Naegleria fowleri*. Any laboratory providing diagnostic parasitology procedures must be prepared to examine these specimens on a stat basis 7 days a week, 24 h a day (orders, specimen collection, processing, examination, and reporting).** Unfortunately, these two procedures can be very difficult to perform and interpret; cross-trained individuals with little microbiology training or experience will find this work difficult and subject to error, and this will cause severe risk management issues for the laboratory. It has been well documented that automated hematology instrumentation lacks the sensitivity to diagnose malaria infections, particularly since most patients seen in an emergency room have a very low parasitemia. **However, even a low parasitemia can be life-threatening in infections with *Plasmodium falciparum* and *Plasmodium knowlesi*.**

Outpatient or Referral Setting

Diagnostic laboratories outside the hospital setting are very appropriate settings for this type of diagnostic testing; the test requests, for the most part, are routine and are batch tested rather than being tested singly. With very few exceptions, stat requests are not relevant and are not sent to such laboratory locations; therefore, immediate testing and reporting are not required.

Decentralized Testing

Point-of-care testing within the hospital (ward laboratories, intensive care units, emergency rooms, and bedside) is usually not considered appropriate for diagnostic parasitology testing; one exception might be the emergency room, where patients with malaria may first present with fever and general malaise. Alternative sites (outpatient clinics, shopping malls, senior citizen groups, and others) are generally not considered appropriate settings for diagnostic parasitology testing, although relevance might be dictated by geographic location and the development of newer, less subjective methods.

Physician Office Laboratories

As mentioned above, the majority of physician office laboratories are not involved in diagnostic parasitology testing; however, as more molecular biology-based (nonmicroscopic) methods are developed, they may become more widely used in this setting. One example is fecal immunoassay methods, specifically designed to detect antigens of *Cryptosporidium* spp., *Giardia lamblia*, the *Entamoeba histolytica*/*Entamoeba dispar* group, and *Entamoeba histolytica*. Rapid tests for the detection of *Plasmodium* spp., particularly *Plasmodium falciparum*, are also currently available (one is FDA cleared in the United States). However, it is critical that the pros and cons of these tests be thoroughly understood prior to patient testing and reporting.

Over-the-Counter (Home Care) Testing

Currently, no diagnostic tests for medical parasitology are available for this potential market. However, outside the United States, some of these options are more likely to be available (none are currently FDA cleared).

Field Sites

Field sites are very relevant for diagnostic parasitology testing, particularly in many areas of the world where instrumentation and automation are not routinely found within clinical laboratories. As the methodology becomes less expensive and easier to use and interpret, testing sites outside the routine laboratory may become more relevant, particularly when associated with epidemiologic studies.

What Factors Should Precipitate Testing?

Travel and Residence History

Although travel history is generally considered in terms of weeks or months, a number of parasitic infections involve potential exposure many years earlier. The patient may become symptomatic years after having left the area of endemic infection. Therefore, it is important to consider long-range history, as well as the previous few weeks or months. This is particularly true when one is considering various places where the patient may have lived prior to becoming symptomatic. The more information the laboratory has regarding past organism exposure, the more likely it is that the causative agent will be identified and the infection confirmed. **It is often imperative that the laboratory follow up with specific questions for the physician; routine information received with the test request may be minimal, at best.**

Immune Status of the Patient

Certain parasites can cause severe illness in debilitated patients and should be considered when these patients present with relevant symptoms. Infections that may cause few or no symptoms in an immunocompetent host may cause prolonged illness or death in an immunocompromised patient. Unfortunately, information regarding the patient's immune status is not always readily available. Client education can help to increase awareness of possible infections with human parasites.

Clinical Symptoms

When infectious diseases are suspected, there are a number of possible etiologic agents. When a patient presents with gastrointestinal symptoms, it is difficult to tell whether the cause is infectious and, if so, which microbe might be responsible. These symptoms often have many noninfectious causes, so laboratory findings can be extremely valuable in confirming a suspected infectious organism. The same diagnostic procedures can also be used to rule out specific etiologic agents.

Documented Previous Infection

Many parasitic infections are difficult to cure or may not cause symptoms on a continual basis; information regarding past exposure or prior documented disease will be valuable for the laboratory. Knowing that the patient may be experiencing a relapse can guide the laboratory in detecting the suspected organism.

Contact with Infected Individuals

In situations where multiple reports of symptomatic patients are confirmed, contacts of these infected patients should be tested, particularly during a potential outbreak. An example of this type of situation is outbreaks of diarrhea in the nursery school setting. When *Cryptosporidium* is identified as the causative agent, all nursery school attendees, employees of the school, and family members are often tested for infection. Another example involves a group of individuals who experience the same symptoms at similar times after attending a function where food was served; the causative agent might be confirmed as *Cyclospora cayetanensis*. These situations have public health significance within the community.

Potential Outbreak Testing

In potential outbreak situations, laboratories that perform certain tests on request only may revise their protocol and begin to test all specimens for a particular suspected parasite. A potential *Cryptosporidium* outbreak might require a change from testing on request only to screening all fecal specimens submitted for parasite testing for this particular organism. Often this occurs after consultation among various groups, such as health care providers, public health personnel, water company personnel, and pharmacy purchasing agents (who may report an increase in the purchase of antidiarrheal medication).

Occupational Testing

The most common example of occupational testing involves food handlers and routine testing for intestinal parasites. This practice is less common than in the past, probably due to financial constraints. Each city, county, and state has specific regulations and/or recommendations.

Therapeutic Failure

With few exceptions, patients are generally retested after therapy to confirm therapeutic efficacy. If testing reveals that the infection has not been eradicated, there may be several reasons. In some cases, the patient may not have taken the medication correctly or may have failed to take the recommended number of doses; these reasons are more likely than the presence of a drug-resistant organism. However, there are certainly examples where drug resistance is possible and is the more likely reason, often depending on the geographic area involved. Another reason might involve the timing between therapy and posttreatment checks for cure; if the time lag is extended, the patient's infection may also represent reinfection. This is particularly true if the patient's living conditions, site, potential parasite and/or vector exposure, and other epidemiological considerations are not modified.

What Testing Should Be Performed?

Routine Tests

"Routine" can imply a widely used, well-understood laboratory test; it can also imply a low- or moderate-complexity method, rather than a high-complexity procedure. Routine diagnostic parasitology procedures could include the ova and parasite examination (O&P exam), preparation and examination of blood films and pinworm tapes or paddles, occult-blood tests, and examination of specimens from other body sites (urine, sputum, duodenal aspirates, urogenital specimens, etc.).

The selection and use of routine test procedures often depend on a number of factors, including geographic area, population served, overall positivity rate, client preference,

number of test orders, staffing, personnel experience, turnaround time requirements, epidemiology considerations, clinical relevance of test results, and cost. Routine tests generally have a wide range of both sensitivities and specificities. As an example, the O&P exam (which involves direct wet mount, concentration, and permanent staining of the smear) could be considered a routine test method for the detection of a number of different intestinal protozoa and helminth infections; this procedure is moderately sensitive but relatively nonspecific. Monoclonal antibody-based test methods tend to be very specific (generally for a single organism, such as *Giardia lamblia*) and more sensitive than the routine O&P exam for specific intestinal protozoa. However, the test results are limited in scope; either the organism is present or it is not, and none of the other possible etiologic agents have been ruled in or out.

Diagnostic laboratories generally offer tests on request; an example is testing for the presence of *Cryptosporidium* spp. However, if a potential waterborne outbreak was suspected, this laboratory might change its approach and begin testing all stool specimens submitted for an O&P exam rather than testing only specimens accompanied by a specific test request for *Cryptosporidium* spp. These decisions require close communication with other entities, as described above for potential outbreak testing.

Special Testing and Reference Laboratories

Special procedures, such as parasite culture, are usually performed in limited numbers of reference laboratories. These procedures require the maintenance of positive control cultures used for quality control checks on all patient specimens; they also require special expertise and time. Many clinical laboratories do not meet these requirements. Although some standardized reagents are now commercially available, many clinical laboratories choose to send their requests for serologic testing for parasitic diseases to other laboratories. Often, the Centers for Disease Control and Prevention (CDC) performs serologic testing on specimens submitted to a given state's department of public health. Generally, specimens for parasitic serologic testing are not submitted directly to CDC but instead are submitted through state public health laboratories. In an emergency situation, consultation with the county or state public health laboratory may allow shipment of a specimen directly to CDC.

Specialized Referral Test Options—DPDx and Other Sites

DPDx (https://www.cdc.gov/dpdx/index.html; accessed 8 June 2019) is a website developed and maintained by CDC's Division of Parasitic Diseases and Malaria. This site provides an interactive and rapid exchange of information with two primary functions. The first function is a reference and training function encompassing parasite reviews, collection, and shipping of clinical specimens. The second function is diagnostic assistance.

Many consultants now provide identification assistance via transmitted microscopic images. There is a wide group of colleagues that share these inquiries and provide input to the requestor. Many of these individuals respond to routine questions with no charge. We all learn from this experience. Many sites also include extensive case histories (Lynne Garcia) (www.med-chem.com [accessed 8 June 2019]; also see the Medical Chemical Corporation site option, Para-Site Online). Another option is the Parasite Wonders blog by Dr. Bobbi Pritt (https://parasitewonders.blogspot.com; accessed 4 July 2020). Other sites can be located by searching "parasite case studies."

Other (Nonmicrobiological) Testing

Test results from other procedures performed in a clinical laboratory can be very helpful when one is trying to diagnose a parasitic infection. Specific examples are routine urinalysis, hematology procedures including a complete blood count, and various chemistry profiles. These results often provide supporting data consistent with a suspected parasitic infection.

What Factors Should Be Considered in Development of Test Menus?

Physical Plant

Provided that equipment requirements are met, most clinical laboratory space designed for microbiology procedures can be used for diagnostic parasitology testing. In smaller facilities, this work can be incorporated into a routine microbiology laboratory. Another consideration is the physical location of the laboratory with respect to the source of clinical specimens. If the distance from the collection site to the laboratory is a consideration, the use of appropriate specimen preservatives must be incorporated into patient specimen collection protocols to ensure that accurate laboratory results will be obtained.

Client Base

Recognition and identification of groups of clients served may dictate the methods and range of diagnostic testing available. Requests for testing for parasites may be minimal in a hospital setting where many procedures are related to elective surgery. In contrast, test requests originating from a large medical center with extensive outpatient clinics may require a broader range of testing and expertise.

Customer Requirements and Perceived Levels of Service

Depending on the client base, patient complexity, history of test requests, and physician interests, the laboratory may be required to provide minimal testing that includes the most commonly performed parasitology procedures. This type of laboratory would generally not be considered a consultative resource; it would need to identify a consultative laboratory to assist with more unusual tests and/or test interpretations. The range and complexity of available tests would also depend on the laboratory's definition of its role in the local, regional, national, or international health care arena.

Personnel Availability and Level of Expertise

Most procedures performed in the diagnostic parasitology laboratory require extensive microscopy training and experience. They are categorized as high-complexity tests by CLIA '88 and are frequently performed by licensed technologists. Based on microscopy examinations, these procedures require a great deal of interpretation. Although cross-training provides some help with certain procedures, including specimen processing, the necessary interpretive skills are not learned in a week or two and can be easily lost without practice. For this reason, it is important to have a minimum of one person who is not only skilled at performing the procedures but also capable of interpreting the findings and providing client training and consultation.

Equipment

The level of equipment required for diagnostic parasitology work is minimal; however, the one expense that should not be limited is that of one or more microscopes with good optics.

Each microscope should be equipped with high-quality (flat-field) objectives (10×, 40×, 50×, 60×, and 100× oil immersion objectives). The oculars should be a minimum of 10×.

Depending on the range of immunoassay testing available, a fluorescence microscope or enzyme immunoassay reader might be desirable. The availability of this equipment varies tremendously from one laboratory to another, and the equipment may be shared with other groups within the laboratory.

Another option is a fume hood, in which the staining could be performed; this is not required, but it is recommended, particularly if the laboratory is still using xylene for dehydration of permanent stained fecal smears.

The rest of the equipment is quite common and can be shared with other areas within the laboratory. Such equipment includes refrigerators, freezers, and pipette systems.

Budget

In general, approximately 70% of a microbiology laboratory budget is related to personnel costs. Although diagnostic procedures in the parasitology area are labor-intensive and may require a microscope with good optics, in general budget costs are minimal. Costs tend to increase when newer immunoassay procedure kits are brought into the laboratory; however, these increased supply costs may be balanced out by diminished labor costs. Each laboratory will have to decide which procedures to offer, which tests can be performed in a batch mode, how many procedures will be ordered per month, what length of turnaround time is required, whether stat testing is possible, and what options exist for referral laboratories, as well as taking educational initiatives and client preferences into consideration.

Although diagnostic parasitology can be an important part of the microbiology laboratory, it is just one section within the total laboratory context and should be analyzed as such for cost containment and clinical relevance. As more automated molecular methods become available, multiple-organism panel testing costs and clinical relevance will need to be considered.

Risk Management Issues Associated with Stat Testing

There are two circumstances in diagnostic medical parasitology that represent true stat testing situations (encompassing orders, specimen collection, processing, examination, and reporting). One is a suspected case of primary amebic meningoencephalitis (PAM) caused by *Naegleria fowleri* or granulomatous amebic encephalitis (GAE) caused by *Acanthamoeba* spp., *Balamuthia mandrillaris*, or *Sappinia diploidea*, and the other situation is any case where thick and thin blood films are requested for testing for blood parasites, possibly those that cause malaria. Extensive discussions of these organisms can be found in reference 15. These tests need to be available on a 24-h/day, 7-days/week basis.

Primary Amebic Meningoencephalitis

Amebic meningoencephalitis caused by *N. fowleri* is an acute, suppurative infection of the brain and meninges. With extremely rare exceptions, the disease is rapidly fatal in humans. The period between contact with the organism and onset of clinical symptoms, such as fever, headache, and rhinitis, may vary from 2 to 3 days to as long as 7 to 15 days.

The amebae may enter the nasal cavity by inhalation or aspiration of water, dust, or aerosols containing the trophozoites or cysts. The organisms then penetrate the nasal mucosa, probably through phagocytosis of the olfactory epithelium cells, and migrate via

the olfactory nerves to the brain. Data suggest that *N. fowleri* directly ingests brain tissue by producing food cups, or amebostomes, in addition to carrying out contact-dependent cytolysis, which is mediated by a heat-stable hemolytic protein, heat-labile cytolysis, and/or phospholipase enzymes. Cysts of *N. fowleri* are generally not seen in brain tissue.

Early symptoms include vague upper respiratory distress, headache, lethargy, and occasionally olfactory problems. The acute phase includes sore throat, stuffy, blocked, or discharging nose, and severe headache. Progressive symptoms include pyrexia, vomiting, and stiffness of the neck. Mental confusion and coma usually occur approximately 3 to 5 days prior to death. The cause of death is usually cardiorespiratory arrest and pulmonary edema.

PAM can resemble acute purulent bacterial meningitis, and these conditions may be difficult to differentiate, particularly in the early stages. The CSF may have a predominantly polymorphonuclear leukocytosis, increased protein concentration, and decreased glucose concentration like that seen with bacterial meningitis. Unfortunately, if the CSF Gram stain is interpreted incorrectly (identification of bacteria as a false positive), the resulting antibacterial therapy has no impact on the amebae, and the patient usually dies within several days. In recent years, fatal cases of PAM have been associated with the use of neti pot sinus irrigation using nonsterile water (16).

Extensive tissue damage occurs along the path of amebic invasion; the nasopharyngeal mucosa shows ulceration, and the olfactory nerves are inflamed and necrotic. Hemorrhagic necrosis is concentrated in the region of the olfactory bulbs and the base of the brain. Organisms can be found in the meninges, perivascular spaces, and sanguinopurulent exudates.

Clinical and laboratory data usually cannot be used to differentiate pyogenic meningitis from PAM, so the diagnosis may have to be reached by a process of elimination. A high index of suspicion is often mandatory for early diagnosis. **All aspects of diagnostic testing (ordering, specimen collection, processing, examination, and reporting) should be carried out stat.** Although most cases are associated with exposure to contaminated water through swimming or bathing, this is not always the case. The rapidly fatal course of 3 to 6 days after the beginning of symptoms (with an incubation period of 1 day to 2 weeks) requires early diagnosis and immediate chemotherapy if the patient is to survive.

Analysis of the CSF shows decreased glucose and increased protein concentrations. Leukocyte counts may range from several hundred to >20,000 cells per mm^3. Gram stains and bacterial cultures of CSF are negative; however, the Gram stain background can incorrectly be identified as bacteria, thus leading to incorrect therapy for the patient.

A definitive diagnosis could be made by demonstration of the amebae in the CSF or in biopsy specimens. Either CSF or sedimented CSF should be placed on a slide under a coverslip and observed for motile trophozoites; smears can also be stained with Wright's or Giemsa stain. CSF, exudate, or tissue fragments can be examined by light microscopy or phase-contrast microscopy. Care must be taken not to mistake leukocytes for actual organisms or vice versa. It is very easy to confuse leukocytes and amebae, particularly when one is examining CSF by using a counting chamber, hence the recommendation to use a regular slide and coverslip. Motility may vary, so the main differential characteristic is the spherical nucleus with a large karyosome.

Specimens should never be refrigerated prior to examination. When the CSF is centrifuged, low speeds (250 × *g*) should be used so that the fresh, unpreserved trophozoites are not damaged. Although bright-field microscopy with reduced light is acceptable, phase

microscopy, if available, is recommended. Use of smears stained with Giemsa or Wright's stain or a Giemsa-Wright's stain combination can also be helpful. If *N. fowleri* is the causative agent, only trophozoites are normally seen. If the infecting organism is *Acanthamoeba* spp., cysts may also be seen in specimens from individuals with central nervous system (CNS) infection. Unfortunately, most cases are diagnosed at autopsy; confirmation of these tissue findings must include culture and/or special staining with monoclonal reagents in indirect fluorescent-antibody procedures. Organisms can also be cultured on nonnutrient agar plated with *Escherichia coli*.

In cases of presumptive pyogenic meningitis in which no bacteria are identified in the CSF, the computed tomography appearance of basal arachnoiditis (obliteration of basal cisterns in the precontrast scan with marked enhancement after the administration of intravenous contrast medium) should alert the staff to the possibility of acute PAM.

The amebae can be identified in histologic preparations by indirect immunofluorescence and immunoperoxidase techniques. The organism in tissue sections looks very much like an *Iodamoeba bütschlii* trophozoite, with a very large karyosome and no peripheral nuclear chromatin; the organisms can also be seen with routine histologic stains.

Organisms can be cultured on nonnutrient agar plated with *E. coli* (15). The trophozoites begin feeding on bacteria and grow to cover the agar surface in 1 to 2 days at 37°C. The presence of the protozoa can be confirmed by examining the agar surface using an inverted microscope or with a conventional microscope by inverting the plate on the stage and focusing through the agar with a 10× objective (Figure 1.1). *N. fowleri*, the causal agent of PAM, undergoes transformation to a pear-shaped flagellate, usually with two flagella but occasionally with three or four flagella; the flagellate stage is a temporary nonfeeding stage and usually reverts to the trophozoite stage (Figure 1.2). *N. fowleri* trophozoites are typically ameba-like and move in a sinuous way. They are characterized by a nucleus with a centrally located, large nucleolus. The trophozoites are also characterized by the presence of a contractile vacuole that appears once every 45 to 50 s and discharges its contents. The contractile vacuole looks like a hole or a dark depression inside the trophozoite and can be easily seen upon examination of the plate under the 10× or 40× objective. When the food supply is exhausted, *N. fowleri* trophozoites differentiate into spherical, smooth-walled cysts (15).

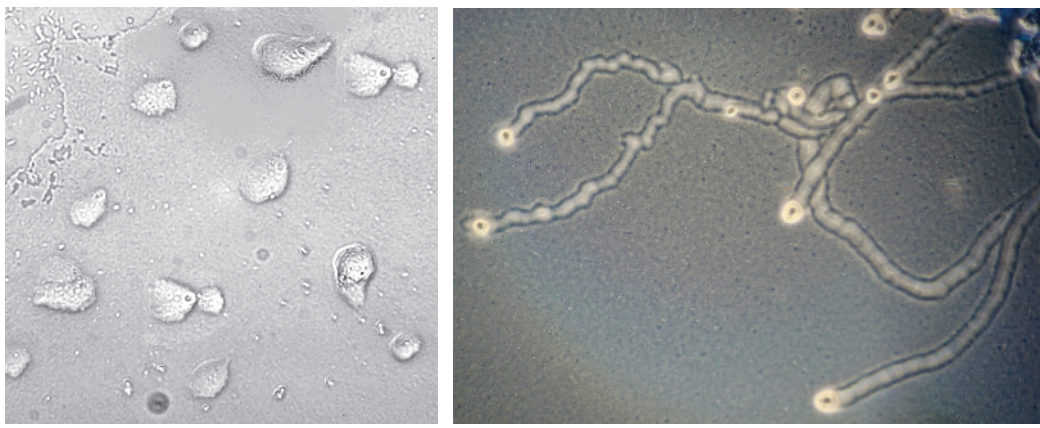

Figure 1.1 Free-living amebae on nonnutrient agar seeded with *E. coli*. Left, trophozoites; right, motility tracks on an agar plate. (Courtesy of Lillian Fritz-Laylin, UMass-Amherst, Amherst, MA.)

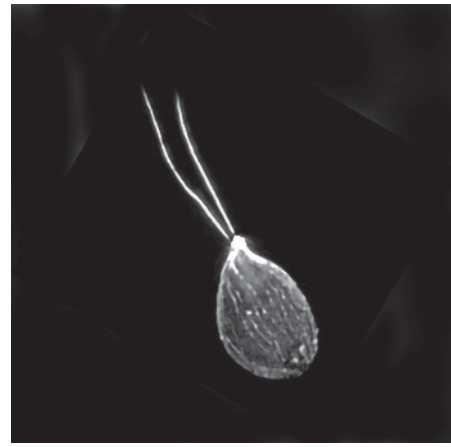

Figure 1.2 When placed in distilled water (enflagellation test), *N. fowleri*, the causal agent of PAM, undergoes transformation to a pear-shaped flagellate, usually with two flagella but occasionally with three or four flagella; the flagellate stage is a temporary nonfeeding stage and usually reverts to the trophozoite stage. Reprinted from Fritz-Laylin LK, Ginger ML, Walsh C, et al, Res Microbiol **162**(6):607–618, 2011, with permission from Elsevier, Ltd.

Granulomatous Amebic Encephalitis and Amebic Keratitis

The most characteristic feature of *Acanthamoeba* spp. is the presence of spine-like pseudopods called acanthapodia. Several species of *Acanthamoeba* (*A. culbertsoni*, *A. castellanii*, *A. polyphaga*, *A. astronyxis*, *A. healyi*, and *A. divionensis*) cause GAE, primarily in immunosuppressed, chronically ill, or otherwise debilitated persons. These patients tend to have no relevant history involving exposure to recreational freshwater. *Acanthamoeba* spp. also cause amebic keratitis, and it is estimated that to date approximately 1,000 cases of *Acanthamoeba* keratitis have been seen in the United States.

GAE caused by freshwater amebae is less well defined and may occur as a subacute or chronic disease with focal granulomatous lesions in the brain. The route of CNS invasion is thought to be hematogenous, with the primary site being the skin or lungs. In this infection, both trophozoites and cysts can be found in the CNS lesions. An acute-onset case of fever, headache, and pain in the neck preceded by 2 days of lethargy has also been documented. The causative organisms are probably *Acanthamoeba* spp. in most cases, but it is possible that others are involved, such as *Balamuthia mandrillaris* and *Sappinia diploidea*.

Cases of GAE have been found in chronically ill or immunologically impaired hosts; however, some patients apparently have no definite predisposing factor or immunodeficiency. Conditions associated with GAE include malignancies, systemic lupus erythematosus, human immunodeficiency virus (HIV) infection, Hodgkin's disease, skin ulcers, liver disease, pneumonitis, diabetes mellitus, renal failure, rhinitis, pharyngitis, and tuberculosis. Predisposing factors include alcoholism, drug abuse, steroid treatment, pregnancy, hematologic disorders, AIDS, cancer chemotherapy, radiation therapy, and organ transplantation. This infection has become more widely recognized in AIDS patients, particularly those with a low $CD4^+$ cell count.

Laboratory examinations similar to that for *N. fowleri* can be used to recover and identify these organisms; the one exception is recovery by culture, which has not proven to be as effective with GAE patients infected with *B. mandrillaris*.

Request for Blood Films

Malaria is one of the few parasitic infections considered immediately life-threatening, and a diagnosis of *P. falciparum* or *P. knowlesi* malaria should be considered to indicate a medical emergency, because the disease can be rapidly fatal. Any laboratory providing the expertise to identify malarial parasites should do so on a stat basis (24 h/day, 7 days/week).

Patients with malaria can present for diagnostic blood work when they are least expected. Laboratory personnel and clinicians should be aware of the stat nature of such requests and the importance of obtaining some specific patient history information. On microscopic examination of the blood films, the typical textbook presentation of various *Plasmodium* morphologies may not be seen by the technologist. The smears should be examined at length and under oil immersion. The most important thing to remember is that even though a low parasitemia may be present on the blood smears (in patients with no prior exposure to malaria and in the presence of residual antibody), the patient may still be faced with a serious, life-threatening disease.

It is important for both physicians and laboratorians in areas where malaria is not endemic to be aware of the problems associated with malarial diagnosis and to remember that symptoms are often nonspecific and may mimic other medical conditions. Physicians must recognize that travelers are susceptible to malarial infection when they visit a country where malaria is endemic and that they should receive prophylactic medication.

With the tremendous increase in the number of people traveling from the tropics to malaria-free areas, the number of imported malaria cases is also on the rise. There have been reports of imported infected mosquitoes transmitting the infection among people who live or work near international airports. It is also possible that mosquitoes can reach areas far removed from the airports. The resulting illness has been termed "airport malaria," i.e., malaria that is acquired through the bite of an infected anopheline mosquito by persons with apparently no risk factors for the disease. Unfortunately, unless a careful history is obtained, the diagnosis of malaria can be missed or delayed. Tests to exclude malaria should be considered for patients who work or live near an international airport and who present with an acute febrile illness. The potential danger of disseminating the mosquito vectors of malaria via aircraft is well recognized; however, modern disinfection procedures have not yet eliminated the risk of vector transportation. Not only can insects survive nonpressurized air travel, but also, they may be transported further by car or other means after arrival at the airport.

We usually associate malaria with patients having a history of travel within an area where malaria is endemic. However, other situations that may result in infection involve the receipt of blood transfusions, use of hypodermic needles contaminated by prior use (for example, by intravenous-drug users), possibly congenital infection, and transmission within the United States by indigenous mosquitoes that acquired the parasites from imported infections. Also, for a number of different reasons, organism recovery and subsequent identification are frequently more difficult than the textbooks imply. It is very important that this fact be recognized, particularly when one is dealing with a possibly fatal infection with *P. falciparum*. It is important to ensure that clinicians are familiar with the following issues.

Automated Instrumentation

Potential diagnostic problems with the use of automated hematology differential instruments have been reported. Some cases of malaria, as well as *Babesia* infection, have been

completely missed by these methods. The number of fields scanned by a technologist on instrument-read smears is quite small; thus, failure to detect a low parasitemia is almost guaranteed. In cases of malaria and *Babesia* infection, after diagnosis had been made on the basis of smears submitted to the parasitology division of the laboratory, all previous smears examined by the automated system were reviewed and found to be positive for parasites. Failure to make the diagnosis resulted in delayed therapy. These instruments are not designed to detect intracellular blood parasites, and the inability of the automated systems to discriminate between uninfected erythrocytes and those infected with parasites may pose serious diagnostic problems in situations where the parasitemia is ≤0.5%.

Patient Information

When requests for malarial smears are received in the laboratory, some patient history information should be made available to the laboratorian. This history can be obtained by asking the ordering physician important questions such as the following:

1. Where has the patient been, and what was the date of return to the United States? (Where do you live and where do you work? [relevant for detecting airport malaria])
2. Has malaria ever been diagnosed in the patient before? If so, which species was identified?
3. What medication (prophylaxis or otherwise) has the patient received, and how often? When was the last dose taken?
4. Has the patient ever received a blood transfusion? Is there a possibility of other transmission via needle (drug user)?
5. When was the blood specimen drawn, and was the patient symptomatic at the time?
6. Is there any evidence of a fever periodicity?

Answers to such questions may help eliminate the possibility of infection with *P. falciparum, P. knowlesi,* or *Plasmodium vivax* (which can cause serious sequelae), usually the only three species that can cause severe disease and, in the case of *P. falciparum* or *P. knowlesi*, can rapidly lead to death.

Conventional Microscopy

Often, when the diagnosis of malaria is considered, only a single blood specimen is submitted to the laboratory for examination; however, **single films or specimens cannot be relied upon to exclude the diagnosis,** especially when partial prophylactic medication or therapy is used. Partial use of antimalarial agents may be responsible for reducing the numbers of organisms in the peripheral blood and lead to a blood smear that contains few organisms and a conclusion that reflects a low parasitemia when in fact serious disease is present. Patients with a relapsing case or an early primary case can also have few organisms in the blood smear. It is recommended that both thick and thin blood films be prepared immediately, and at least 300 oil immersion fields should be examined on both films before a negative report is issued. Since one set of negative smears does not rule out malaria, additional blood specimens should be examined over a 36-h period. **Although Giemsa stain has been recommended for all parasitic blood work, the organisms can also be seen if other blood stains, such as Wright's stain or any of the rapid blood stains, are used.** Blood collected with the use of EDTA anticoagulant is preferred over heparin; however, if the blood remains in the tube for approximately an hour or more, true stippling might not be visible within the infected erythrocytes (e.g., those infected with

P. vivax). When EDTA is being used, if blood is held for more than 2 h prior to blood film preparation, several artifacts may be seen; after 4 to 6 h, some of the parasites will be lost. During the time when the parasites are in the tube of blood, they continue to grow and change according to the life cycle for that species. Also, when anticoagulants are used, it is important to remember that the proper ratio of blood to anticoagulant is necessary for good organism morphology—the tube should be filled with blood. Both thick and thin blood films should be prepared immediately after receipt of the blood. If the specimen is sent to a reference laboratory, both the thick and thin blood films, as well as the tube of blood (room temperature), should be sent. Since this test is always considered a stat request, it is also important to know what turnaround times are available from the reference laboratory.

All requests for malaria diagnosis are considered stat requests, and specimens should be collected, processed, examined, and reported accordingly. Although other diagnostic tests can be ordered, any request for examination of blood films should include a possible diagnosis of malaria; thus, these requests are always considered stat. Not only should the blood collection be considered stat, but also, the processing and examination of both thick and thin blood films should be performed immediately on receipt of the blood. Often, immunologically naive individuals with no prior exposure to malaria can present to the emergency room or clinic with symptoms such as fever and malaise and a relevant travel history to an area of the world where malaria is endemic. These patients can have very vague symptoms, but they have the potential to become very ill with malaria, even with a low parasitemia (0.0005% to 0.1%).

Remember, when reporting results (genus and species), provide the stages of malaria present (rings, developing trophozoites, schizonts, and/or gametocytes); this information is very relevant for those providing therapy for the patient.

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Table 1.1 Common features of the neglected tropical diseases[a]

Ancient afflictions that have burdened humanity for centuries
Poverty-promoting conditions
Associated with stigma
Rural areas of low-income countries and fragile states
No commercial markets for products that target these diseases
Interventions, when applied, have a history of success

[a] See reference 2.