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2

General Principles in the Diagnosis of Infection

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The identification of infection in biopsied tissues is the primary responsibility of the surgical pathologist. In an age when both noninvasive and minimally invasive approaches and techniques have increased, it is important to revisit the role of the biopsy in the diagnosis of infection (Box 2.1). Isolating microorganisms in the microbiology laboratory is a sensitive and accurate approach to their identification, but it has several important limitations. First, it cannot distinguish infection from colonization, nor can it ascertain the significance of the isolated organism. Only the presence of an organism in situ, together with an expected inflammatory response by the host, constitutes acceptable evidence of its role in infection.

For example, consider how to interpret the clinical significance of a fungus isolated from the airways of a patient with bronchiectasis who also has a new pulmonary infiltrate in the setting of immunosuppression. Is the fungal isolate the likely cause of the opportunistic infection, or might it be a benign commensal? Studies have attempted to address this question[1] with guidelines formulated for practice, but these are indeed merely "guidelines" because only identification of a potential pathogen within a site of infection can provide substantive evidence that the fungus is an invasive pathogen. For this and other reasons to be addressed in this text, the pathologic diagnosis of infection is a critical element in formulating optimal therapy.

Sampling

Tissue sampling is fundamentally important in the diagnosis of infection. All excised tissues should be considered as potentially infective. This approach fosters due diligence with respect to the possibility of contagion, as well as thoughtful concern as to how the tissues will be handled to optimize the chances of establishing an accurate diagnosis (Box 2.2). Samples of excised tissues should be harvested by sterile technique and sent to the microbiology laboratory with information concerning the types of organism that are being considered diagnostically. Directions to consider anaerobic and fastidious species should be clearly stated.

The surgical pathologist must ascertain that all diagnostic possibilities have been considered. Consultation with an infectious disease specialist can be invaluable in ensuring that specimens are properly handled ab initio. What must be avoided is thoughtlessly placing a biopsy specimen directly into formalin fixative without first considering a diagnosis of infection.

Touch imprints should be routinely prepared and can be stained in the frozen-section suite or in the microbiology laboratory. In general, 5 to 10 touch imprints will suffice, with sampling from the most suspicious portions of the biopsy specimen (e.g., areas of necrosis or suppuration).

Harvesting a portion of the biopsy specimen for ultrastructural analysis can foster the accurate diagnosis of many organisms (e.g., viruses, *Tropheryma whippeli,* microsporidia).[2] Specimens may be harvested for polymerase chain reaction (PCR) testing to establish the diagnosis of others (e.g., *Coxiella*, *Mycobacterium*, *Rickettsia*).[3]

The rapid diagnosis of a frozen section can help to focus on the diagnostic workup. All of the pertinent histochemical and ancillary studies can ideally be ordered before the permanent sections are processed, to avoid undue delay in diagnosis.

• BOX 2.1 Role of the Surgical Pathologist in the Diagnosis of Infection

Establish morphologic diagnosis of infection
Assess immunocompetence of the host
Narrow the differential diagnosis of possible pathogens
Confirm results of microbiologic cultures
Refute the relevance of microbiologic cultures
Establish diagnosis unrelated to infection
Identify concomitant infection in a primary inflammatory or neoplastic disorder
Identify new pathogens

• BOX 2.2 Optimal Handling of Tissue Biopsies: Always Consider Infection!

Make touch imprints for histochemical staining
Handle samples for microbiologic culture with sterile technique
Harvest samples for ultrastructural examination in glutaraldehyde fixative
Harvest fresh samples for appropriate polymerase chain reaction assays
Freeze portion of biopsy specimen for research
After all of this is done, place biopsy specimen in formalin

Diagnosing Infection In Situ

Because host immune mechanisms can greatly amplify the host response, the actual number of pathogens present in tissues is frequently surprisingly small. This means that many sections may need to be examined before a pathogen is identified. Although few surgical pathologists would balk at the idea of ordering additional sections to exclude malignancy in a biopsy they deemed suspicious, it is not uncommon for a pathologist to examine only a single histochemically stained tissue section in the diagnostic process of infection.[4] More egregious is the fantasy that the causative infectious agent will eventually be diagnosed by the microbiology laboratory, so there is no need for the surgical pathologist to belabor the process.

This approach is wrong minded for several reasons. First, the microbiology laboratory may fail to identify a causative organism.[5] Second, the organism isolated by the laboratory may not represent the actual infective agent in vivo. The analogy is the role for Gram staining of secretions in chronically intubated patients to determine whether there is a neutrophilic exudate consistent with infection and whether there is a predominating organism—steps that can promote the choice of appropriate antibiotic therapy.[6] In this setting, undue emphasis on culture results can lead to a seemingly endless process of adding or eliminating antibiotics in patients who are merely colonized by bacteria and not actually infected. Treatment decisions that do not take into account the host response and dominating organisms will tend to favor the production of increasingly antibiotic-resistant isolates and may potentially compromise public health. This is only one of several compelling reasons to consider diagnostic biopsies in patients with infections in situations that do not readily yield to noninvasive approaches.

Potential Limits of Biopsy Interpretation

Despite the merits of examining biopsy specimens in the diagnosis of infection, one must be aware of those situations in which the sensitivity and specificity of histochemically stained sections are limited. An example is tuberculosis, in which biopsies can fail to demonstrate mycobacteria in almost half of cases.[7] However, even in this setting, the appearance of the inflammatory response in situ should foster a working diagnosis that is often sufficiently reliable to institute empirical treatment.

TABLE 2.1 Tissue Responses to Infection

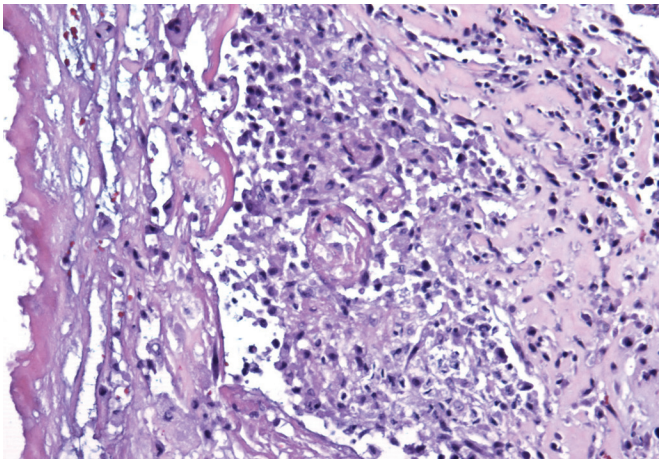
Type of Inflammation	Example
Exudative inflammation	Pyogenic bacteria
Necrotizing inflammation	Gram-negative bacteria, amebiasis
Granulomatous inflammation	Mycobacteria, fungi
Histiocytic inflammation	*Rhodococcus, Legionella,* Whipple disease
Eosinophilic inflammation	Fungi, parasites
Cytopathic changes	Viruses
No response	Host energy

Classification of Patterns of Infection

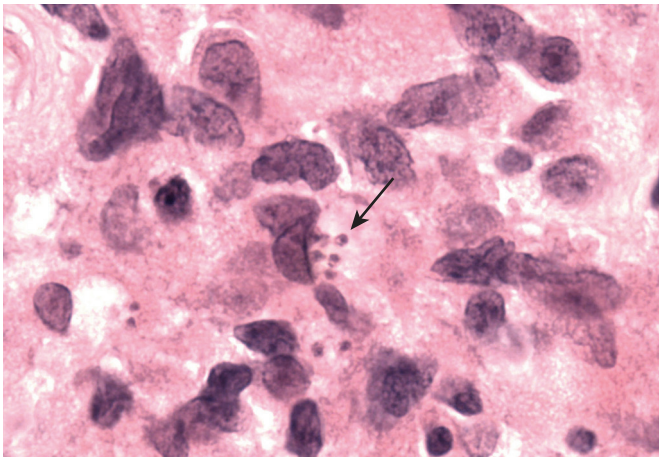
There is currently no uniformly accepted classification schema for the histologic patterns of response yielded by microorganisms. The inflammatory response in infection is a function of the host response, which is in turn a function of (1) the anatomy of the affected organ, (2) the virulence factors produced by the infective agent, and (3) host immunocompetence. The surgical pathologist must be aware that a single species of microorganism may be capable of evoking a variety of different patterns of inflammation. An example is the broad spectrum of disorders produced in response to infection with *Aspergillus* spp., which ranges from benign colonization, to hypersensitivity responses, to malignant angioinvasive infection.[8]

The characteristic types of inflammation elicited by infection (Table 2.1) can be broadly categorized as follows.

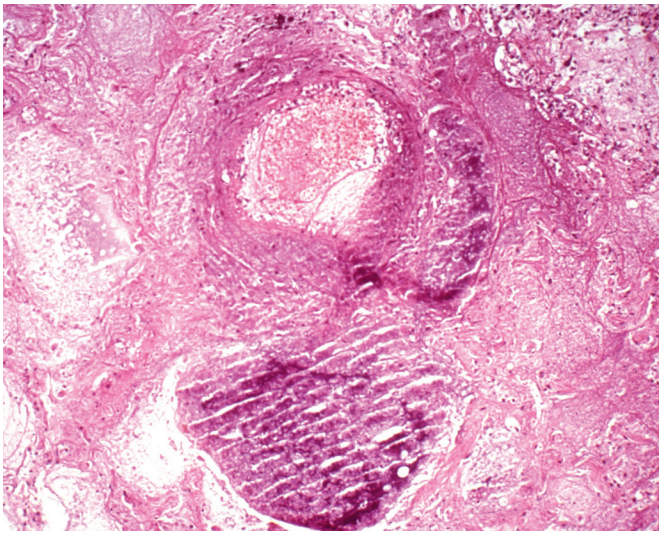
1. **Pyogenic responses.** In these responses, neutrophils predominate, leading to pus formation. They are evoked primarily by bacteria, although viruses and fungi can also elicit them (Fig. 2.1).
2. **Necrotizing inflammation.** Tissue necrosis can occur in several forms. In certain infections, such as those caused by amoebas or gram-negative bacteria, liquefactive necrosis is frequently seen (Fig. 2.2). Other forms, such as ischemic, mummefactive, and caseous necrosis, are often seen in mycobacterial and fungal infections.
3. **Granulomatous inflammation.** This response is characterized by the presence of epithelioid macrophages with multikaryon (giant cell) formation. It appears to reflect cell-mediated immunity to poorly catabolized antigens and is evoked by mycobacteria, fungi, and parasites (Fig. 2.3).
4. **Histiocytic inflammation.** These responses are characterized primarily by the presence of foamy macrophages and are a prominent component of infections caused by *Legionella, Rhodococcus, Calymmatobacterium, Leishmania,* and *T. whippeli* (Fig. 2.4). In patients who are severely immunocompromised,



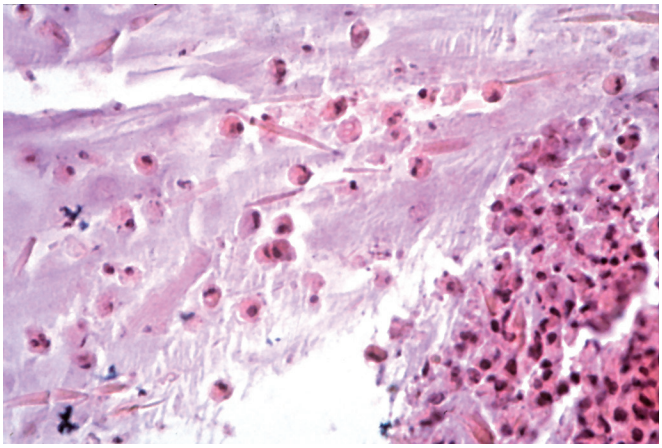
• **Figure 2.1** Pyogenic response in acute infective endocarditis due to *Streptococcus* spp. with neutrophilic exudate (×400).



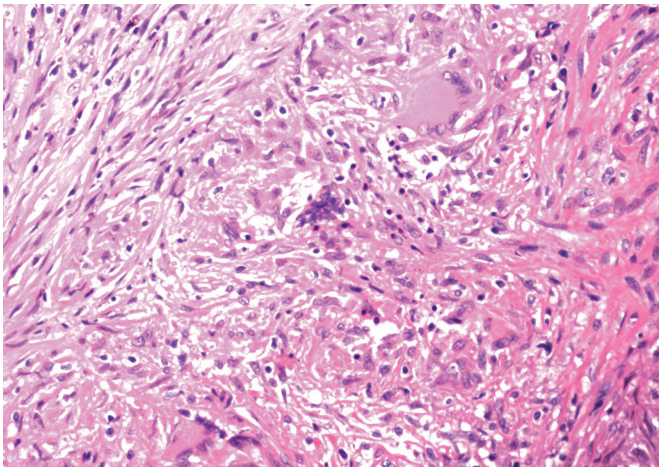
• **Figure 2.4** Histiocytic response shows "foamy" macrophages containing *Leishmania donovani (arrow)* (×600).



• **Figure 2.2** Necrotizing response to *Pseudomonas aeruginosa,* showing liquefactive destruction of lung tissue (×250).



• **Figure 2.5** Eosinophilic response to *Aspergillus fumigatus* (×400).

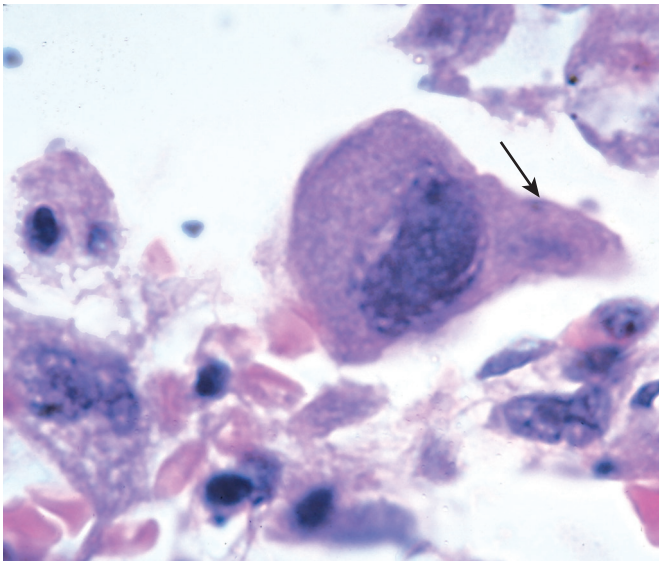


• **Figure 2.3** Granulomatous response to *Mycobacterium tuberculosis* (×25).

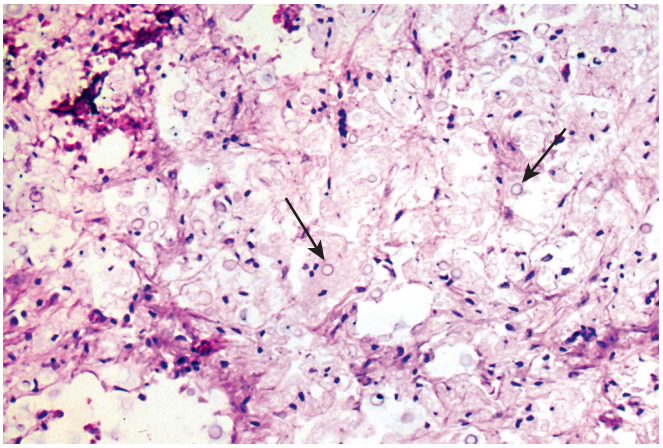
organisms that normally elicit granulomatous inflammation may instead evoke histiocytic infiltrates.

5. **Eosinophilic inflammation.** This is seen in response to multicellular parasites and certain fungi (Fig. 2.5).
6. **Cytopathic changes.** Although this is not properly a type of inflammation, cytopathic changes do reflect a response to viral infection. Nuclear inclusions are part of the response to DNA viruses, whereas cytologic inclusions are seen with some RNA and DNA viral infections, such as cytomegalovirus (Fig. 2.6).
7. **Null responses.** In the setting of profound immunosuppression, one may not see inflammation; only the uninhibited growth of microorganisms is apparent (Fig. 2.7).

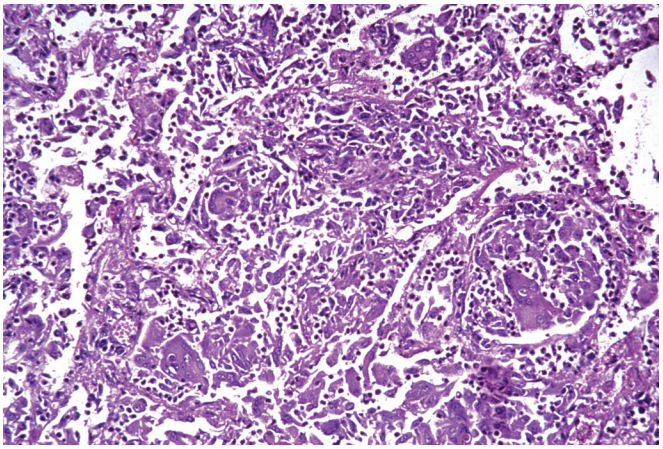
This classification schema is only a crude approximation because overlap patterns of inflammation are common, as with necrotizing granulomatous inflammation, granulohistiocytic inflammation (Fig. 2.8), and granulomatous inflammation with tissue eosinophilia (Fig. 2.9). The primary didactic element is that careful consideration of the histologic response in situ can help to narrow what would otherwise be a very broad differential diagnosis and can also provide invaluable information concerning host immunocompetence. For this reason, surgical pathologists must develop expertise concerning the inflammatory patterns that can accompany reduced immunocompetence resulting from genetic



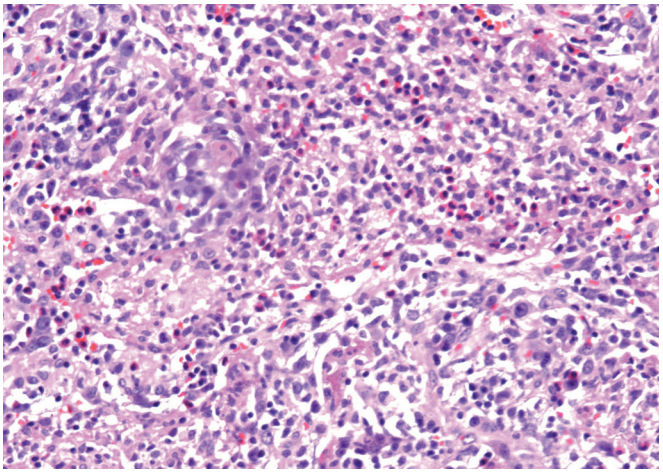
• **Figure 2.6** Cytopathic response to *Cytomegalovirus* with both nuclear and cytoplasmic *(arrow)* inclusions (×600).



• **Figure 2.7** Null response to *Cryptococcus neoformans (arrows)* (×400).



• **Figure 2.8** Granulohistiocytic response to *Blastomyces dermatitidis* (×250).



• **Figure 2.9** Granulomatous response with tissue eosinophilia due to *Coccidioides immitis* (×250).

factors, age, toxins, and drugs, because they can skew the expected pattern of inflammation and at times confound the diagnosis.

Histochemical Stains

The identification of microorganisms in biopsy samples is enhanced by the selective application of widely available histochemical stains (Table 2.2). Pathologists should be aware of the spectrum of histochemical staining by microorganisms and knowledgeable with respect to how to formulate combinations of stains to enhance diagnostic specificity.[9]

Hematoxylin and Eosin

The majority of pathogens can be identified with the standard hematoxylin and eosin (H&E) stain. These include cytopathic viruses, some bacteria, most fungi, and virtually all parasites (Box 2.3).

Gram Stain

The tissue Gram stain is a congener of the Gram stain used routinely to identify organisms in body secretions and fluids. The Brown-Hopps stain is currently the preparation of choice because it enhances gram-negative bacteria and rickettsia to a greater degree than the Brown-Brenn. In addition, the latter can be hazardous to technical personnel and has largely fallen into disfavor. The tissue Gram stain colors the cell walls of gram-positive bacteria a deep violaceous blue (Fig. 2.10A) and gram-negative bacteria a pale salmon pink (see Fig. 2.10B). Consequently, it is far easier to detect gram-positive species, and one must be careful not to overlook the presence of faintly stained gram-negative species. Gram variability is a potential pitfall in interpretation because it can raise the specter of polymicrobial infection. Attention to the uniform morphologic characteristics of stained organisms is the best way to avoid being misled by this phenomenon.

Nonbacterial pathogens can also be identified with the Gram stain. The blastoconidia (yeast) of *Candida* spp. (Fig. 2.11A) and the microconidia of *Aspergillus* spp. (see Fig. 2.11B) are gram positive, and this feature can help in distinguishing these species from other fungi. Microsporidia can be well demonstrated as gram-positive intracellular inclusions within cells (Fig. 2.12).

TABLE 2.2 Histochemical Staining Characteristics of Microbes

Organism	Staining Characteristics
Viruses	
Influenza	No cytopathic change
Coronavirus (SARS)	No cytopathic change
Adenovirus	H&E (smudge cells); IHC
Cytomegalovirus	H&E (intranuclear and cytoplasmic inclusions); IHC; PAS and GMS (intracytoplasmic inclusions)
Herpes virus	H&E (intranuclear inclusions); IHC
Measles	H&E (intranuclear inclusions, polykaryons)
Respiratory syncytial virus	H&E (polykaryons); IHC
Parainfluenza	H&E (intracytoplasmic inclusions)
Bacteria	
Gram positive	Tissue Gram, GMS (all)
Gram negative	Tissue Gram, GMS (some)
Legionella	Silver impregnation
Nocardia	Tissue Gram, GMS, modified ZN
Actinomyces	Tissue Gram, GMS
Mycobacteria tuberculosis	ZN and modified ZN; PCR
Atypical mycobacteria	Mo dified ZN, ± ZN, PCR
Fungi	
Histoplasma	GMS, PAS
Cryptococcus	H&E, GMS, PAS, mucicarmine; Fontana, IHC
Blastomyces	H&E, GMS, PAS, mucicarmine (weak)
Coccidiomyces	H&E, GMS, PAS
Candida	H&E, GMS, PAS, Gram stain; IHC
Aspergillus	H&E, GMS, PAS, IHC
Zygomyces	H&E, GMS, PAS
Pseudeallescheria	H&E, GMS, PAS
Alternaria and dematiaceous fungi	H&E, GMS, PAS, Fontana
Parasites	
Protozoa	H&E, PAS, Gram stain (microsporidia); IHC *(Toxoplasma)*
Metazoans	H&E, trichrome stain
Echinococcus	GMS in chitinous wall, modified ZN (hooklets)
Paragonimiasis	Ova birefringent
Schistosomiasis	Lateral and terminal spines stain with modified ZN

GMS, Gomori methenamine silver stain; *H&E*, hematoxylin and eosin stain; *IHC*, immunohistochemical methods; *PAS*, periodic acid–Schiff stain; *PCR*, polymerase chain reaction; *SARS*, severe acute respiratory syndrome; *ZN*, Ziehl-Neelsen stain.

• BOX 2.3 Microbes That Can Be Identified With Hematoxylin and Eosin Stain

Cytopathic viruses
Bacteria in colonies or in "granules"
Most fungi
Parasites

Silver Impregnation

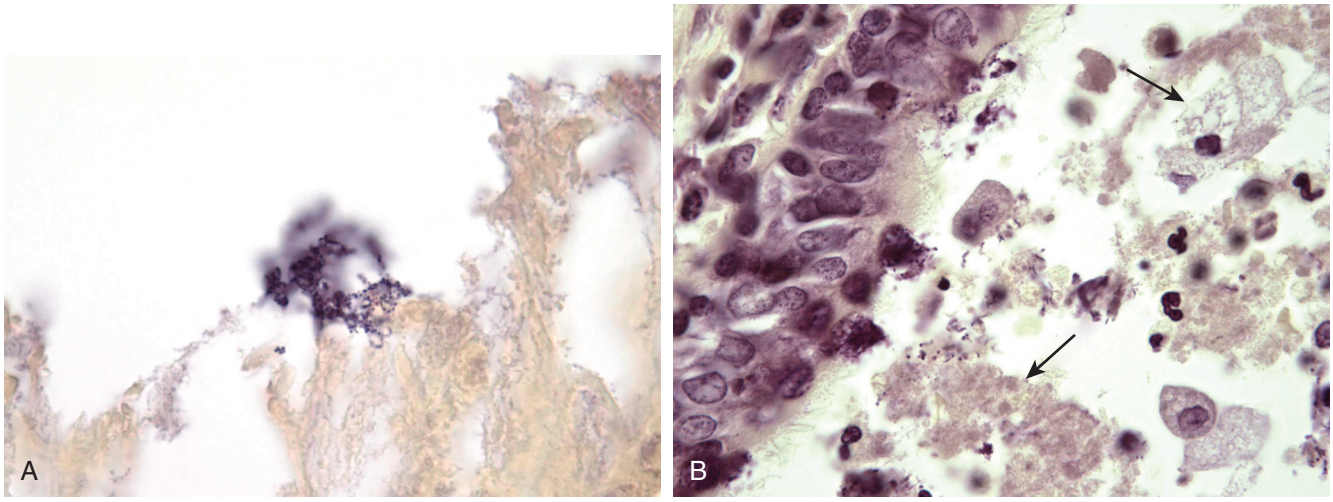
The impregnation of tissue sections with silver constitutes the basis of the Warthin-Starry, Dieterle, and Steiner stains. There is some controversy among experts as to whether these stains are equally efficacious in the identification of certain organisms, such as *Bartonella* spp., but they generally yield comparable results. In theory, all eubacteria, including mycobacteria, will stain positively with silver impregnation. However, in our experience, they do not do so reliably, and this approach cannot be recommended as a screening tool. In general, bacteria are enhanced both colorimetrically and in size by the deposition of silver salts on their cell walls, making them easier to identify but at times causing confusion in interpretation. Background staining presents a problem in interpretation, but the morphologic regularity of eubacteria usually allows for accurate identification after experience has been established with the technique.

Certain weakly gram-reactive or non–gram-reactive bacteria cannot be demonstrated reliably by any other histochemical method. These include *Treponema* spp. (Fig. 2.13), *Borrelia* spp., *Bartonella* spp., *Leptospira* spp., and *Calymmatobacterium*. Weakly staining gram-negative bacteria, including *Legionella* spp., *Burkholderia* spp., *Francisella* spp., and *Helicobacter*, are also best demonstrated by silver impregnation.

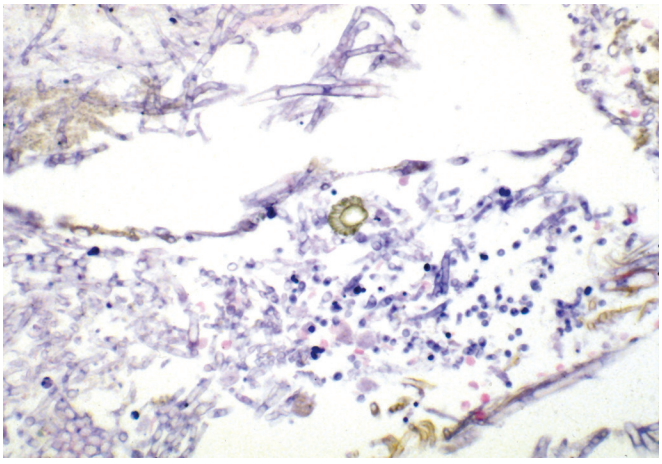
Fungal Stains

The Gomori methenamine silver (GMS) and Gridley stains are the preferred methods for demonstrating fungi (Table 2.3). Because certain fungi demonstrated by GMS do not consistently stain well with periodic acid–Schiff (PAS), the latter should be reserved as a secondary approach, but it can at times enhance morphologic detail. Although the GMS is often counterstained with methyl green for contrast, other counterstains can be applied. For example, it is possible to counterstain with H&E; this allows for a detailed assessment of the cellular immune response and promotes accurate identification of intravascular and perineural invasion by organisms.

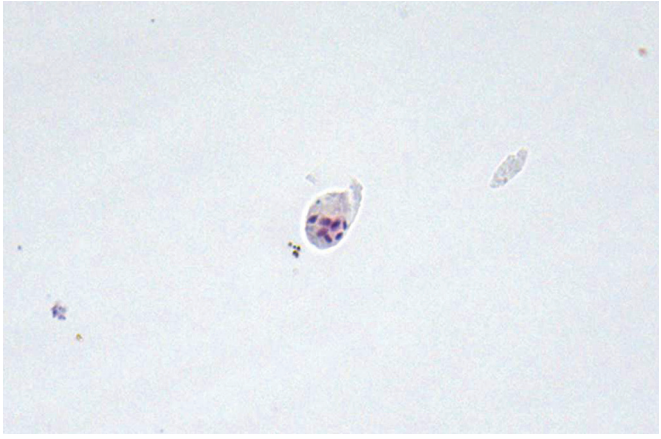
All gram-positive bacteria, including the actinomycetes, stain with GMS (Fig. 2.14), as do some encapsulated gram-negative bacteria, such as *Klebsiella* spp. Bacteria that have been treated before tissue sampling (e.g., infective endocarditis) may not be well decorated by the Gram stain, but they often retain their GMS positivity. For this reason, both stains should be examined before excluding a gram-positive bacterial infection. The actinomycetes, including mycobacteria, are gram-positive eubacteria and consequently also stain with GMS. The GMS is the stain of choice for demonstrating *Pneumocystis jiroveci* (Fig. 2.15), and it highlights the trophozoites of *Entamoeba histolytica*, encysted amoebas, the intracytoplasmic inclusions of cytomegalovirus-infected cells, the polar bodies of microsporidia, and the cyst wall of *Echinococcus* spp. (Fig. 2.16).



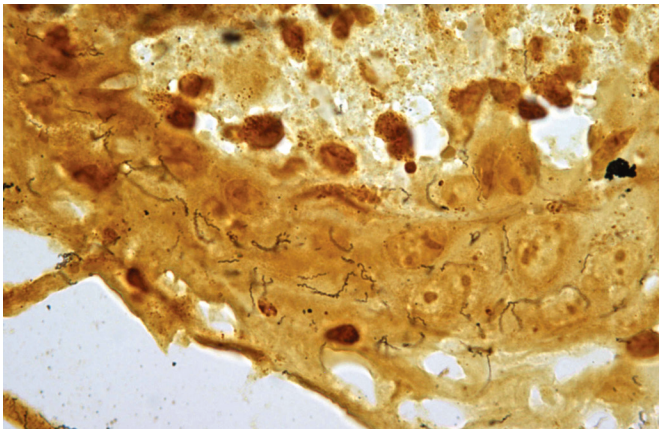
• **Figure 2.10** **A,** *Streptococcus* spp. stain deep blue-magenta (×600). **B,** Gram-negative bacteria are pale salmon-pink *(arrows)* (×600).



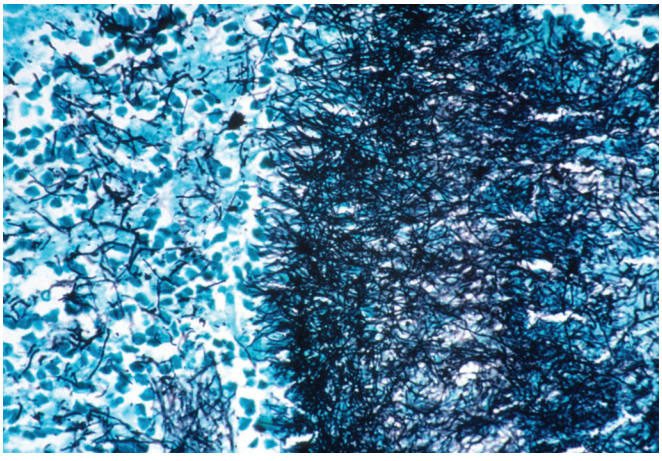
• **Figure 2.11** The microconidia of *Aspergillus fumigatus* stain intensely gram positive (×250).



• **Figure 2.12** Gram-positive intracytoplasmic microsporidia (×400).



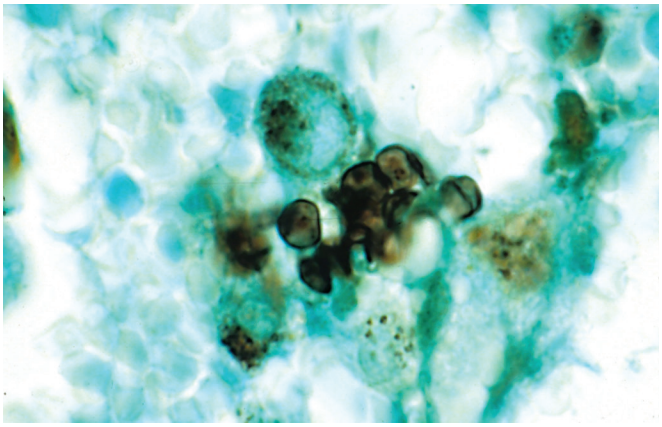
• **Figure 2.13** Spirochetes of *Treponema pallidum* stain with Warthin-Starry silver impregnation (×400).



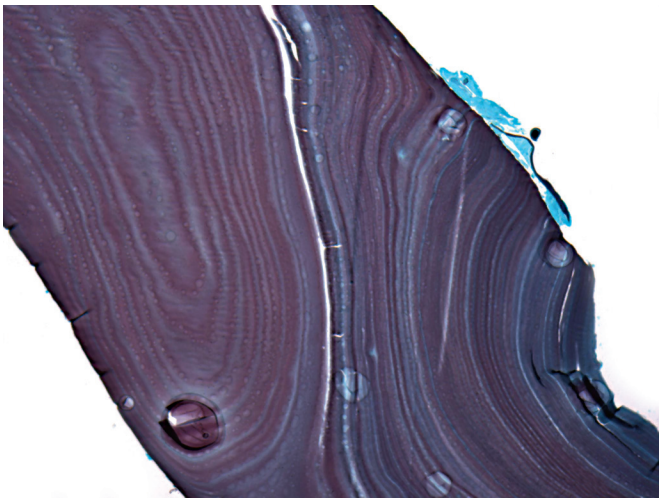
• **Figure 2.14** *Actinomyces israelii* stains with Gomori methenamine silver (×250).

TABLE 2.3 Fungal Identification in Tissue

Organism	Size (Width in µm)	Defining Morphology
Histoplasma capsulatum	2-5	Narrow-neck bud
Cryptococcus neoformans	5-20	Narrow-neck bud
Blastomyces dermatitidis	15-30	Broad-based bud
Candida glabrata	3-5	Budding, no pseudohyphae
Candida spp.	2-3	Yeast, pseudohyphae, hyphae
Aspergillus spp.	3-5	Acute-angle branching, septate, conidial head
Zygomyces spp.	5-8	Right-angle branching, ribbons, pauciseptate
Pseudallescheria spp.	3-4	Acute-angle branch, septate, terminal chlamydospore, pigmented conidia
Fusarium spp.	4-5	Acute and right-angle branch, septate, narrowed branch points
Coccidioides immitis	20-200	Endosporulation



• **Figure 2.15** Gomori methenamine silver-positive cysts of *Pneumocystis jirovecii* (×600).



• **Figure 2.16** Gomori methenamine silver-positive wall of cyst produced by *Echinococcus granulosus* (×200).

Acid-Fast Bacteria Stains

The Ziehl-Neelsen (ZN) stain and its modifications historically have been essential tools in the identification of mycobacteria.[3] *Mycobacterium* spp. (Fig. 2.17A) are also GMS positive (see Fig. 2.17B), and some atypical mycobacteria, such as *Mycobacterium avium-intracellulare* complex (MAC), also stain with PAS. The modified stain for acid-fast bacteria (Fite-Faraco or Putt) detects mycobacterial antigens that are sensitive to strong acid, a step in the decolorization of the ZN stain. For this reason, it can be used to screen for all mycobacteria and may be required to detect certain atypical mycobacteria, such as *Mycobacterium leprae*, as well as *Nocardia*, *Rhodococcus*, and *Legionella micdadei*. The cortical spines of *Schistosoma* spp. (Fig. 2.18), the hooklets of *Echinococcus*, and the spores of *Cryptosporidium* also stain well, but variably, with modified acid-fast bacillus stains.

Connective Tissue Stains

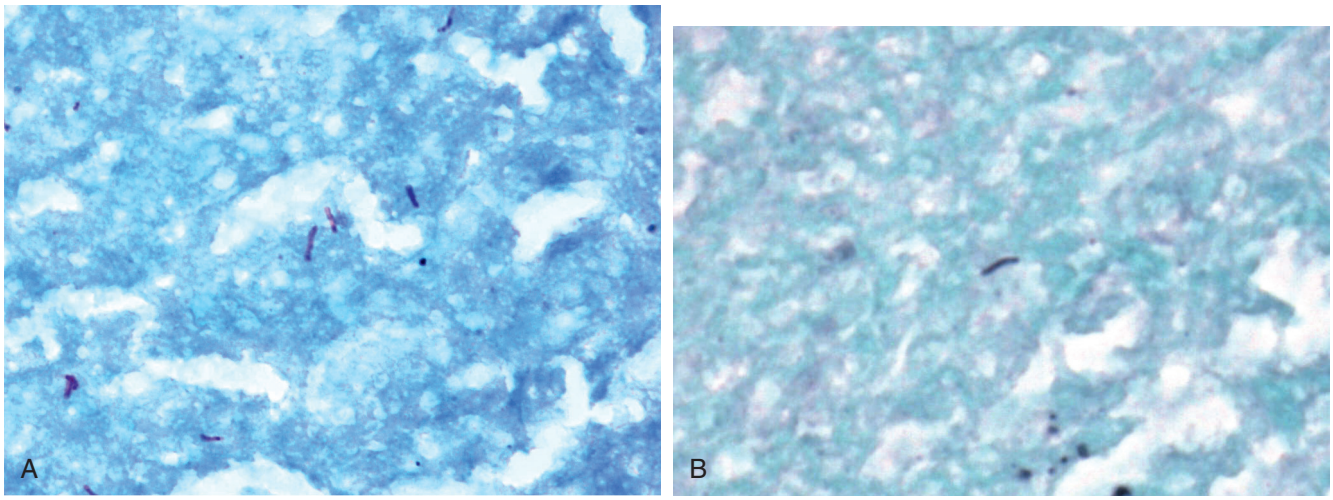
Masson trichrome, Movat pentachrome, and Wilder reticulin stains can be useful ancillary methods for classifying helminthic infections (Fig. 2.19). The inclusions of cytomegalovirus are demonstrated well by trichrome stains. The reticulin stain demonstrates the details of most helminths, the amastigotes of trypanosomes, and the rod-shaped kinetoplast of *Leishmania* spp.

Giemsa Stains

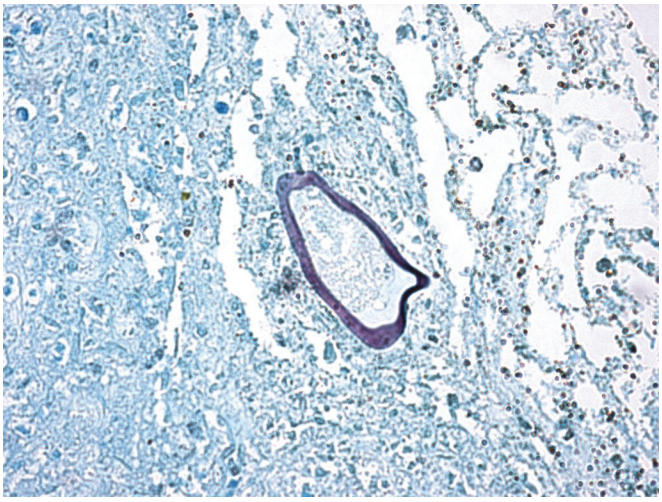
Giemsa stains and their variants can help in identifying a wide spectrum of pathogens, including protozoa, bacteria, chlamydia, and rickettsia. However, the small size of some of these organisms (e.g., rickettsiae) limits the degree of confidence in establishing an accurate diagnosis, and prior experience in diagnosing these infections is essential.[10]

Mucicarmine

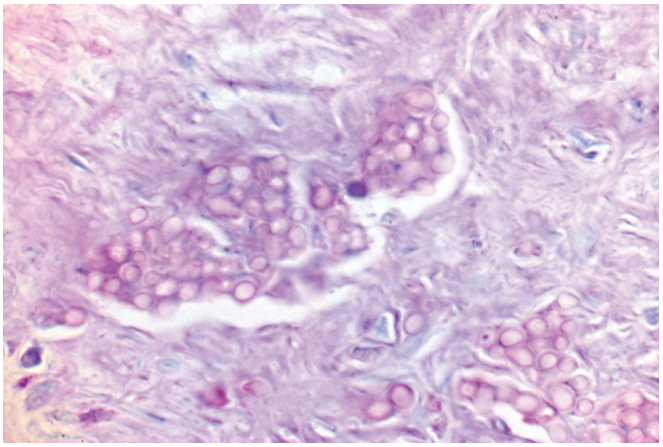
Several fungi, most notably *Cryptococcus*, *Blastomyces*, and *Rhinosporidium,* exhibit mucicarminophilia, either in their secreted capsules *(Cryptococcus)* or in their cell walls. Mucicarminophilia



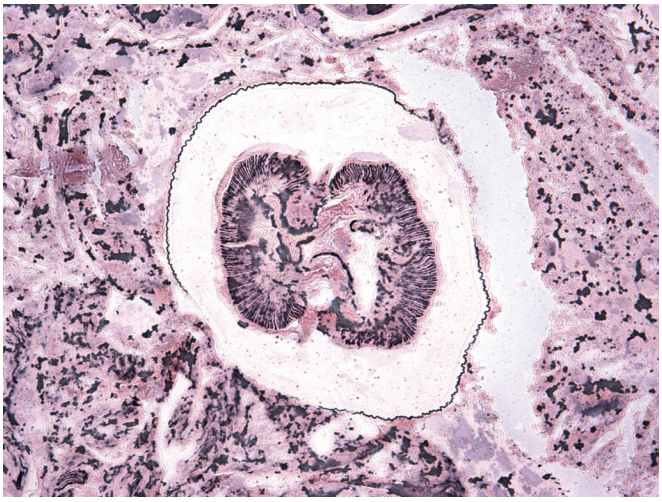
• **Figure 2.17** *Mycobacterium tuberculosis* stains with Ziehl-Neelsen stain (×600) **(A)** and Gomori methenamine silver stain (×600) **(B)**.



• **Figure 2.18** Cortical spine of ovum of *Schistosoma mansoni* stains positive with Fite-Faraco stain (×600).



• **Figure 2.20** Mucicarmine stain decorates the capsule of *Cryptococcus neoformans* (×400).



• **Figure 2.19** Wilder reticulin stain highlights *Dirofilaria immitis* (×150).

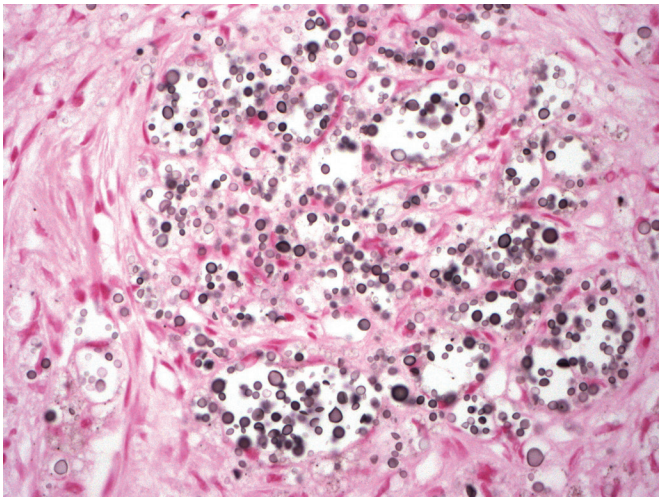
is an essential feature in the diagnosis of *Cryptococcus*; however, this staining, although invariably present, may be difficult to detect in capsule-deficient variants (Fig. 2.20).[11]

Melanin Stains

The Fontana-Masson stain demonstrates premelanin precursors within the cell wall of *Cryptococcus neoformans* and is an essential confirmatory approach to the in situ identification of capsular-deficient forms (Fig. 2.21).[12] All dematiaceous fungi are positive with Fontana-Masson, and this stain can help to confirm the impression of pigmentation seen in H&E sections.

Viral Inclusion Body Stains

A number of stains (e.g., Feulgen) can detect viral inclusions with cells. However, none add considerably to the H&E stain in this regard, and they are rarely adopted in practice, especially since confirmatory immunostains have become more widely available.



• **Figure 2.21** Fontana-Masson stain assists in the identification of an "acapsular" variant of *Cryptococcus neoformans* (×400).

TABLE 2.4 Immunohistochemical Stains Commercially Available for Microbe Identification in Paraffin-Embedded Tissues

Fungi	Viruses and Bacteria
Aspergillus (genus only)	Herpes virus 1 (cross-reacts herpes virus 2)
Cryptococcus	Varicella zoster
Histoplasma	Cytomegalovirus
Candida spp.	Respiratory syncytial virus
Coccidioides immitis	Adenovirus
Pneumocystis jiroveci	Epstein-Barr (Epstein-Barr-encoded RNA)
Pseudallescheria boydii	Actinomycetes
Zygomycoses (genus only)	*Actinomyces israelii*
Sporothrix schenckii	*Actinomyces naeslundii*
Trichosporon	*Arachnia propionica*

Immunohistochemical Methods

A large number of immunostains are available that can be helpful in the identification of microorganisms (Table 2.4).[13] Many of these are commercially available and currently enjoy wide popularity in diagnostic pathology laboratories. Others are available reliably only at highly specialized centers, such as the Centers for Disease Control and Prevention. Development of new immunohistochemical techniques can be a worthwhile but labor-intensive task. Because there is wide cross-reactivity among fungal species and among bacteria, it is critical to establish the potential cross-reactivity of any new antibody and its relative specificity.[14] Nevertheless, it may at times be possible to limit the use of a reagent to a narrow range of differential diagnostic possibilities (e.g., to distinguish *Aspergillus* spp. from *Pseudallescheria boydii* infection).

Molecular Diagnostics

Molecular techniques continue to dramatically reshape clinical microbiology practice. Currently, molecular techniques involving the identification of microbial nucleic acids are critical to the management of a growing number of infectious agents, most importantly the chronic viral infections, including human immunodeficiency virus (HIV), hepatitis B virus, and hepatitis C virus. HIV management is a prototype for the implementation of molecular medicine because the diagnosis can be made using reverse transcriptase–polymerase chain reaction (RT-PCR) amplification of viral RNA, antiviral therapy regimens can be adjusted based on serial RT-PCR viral load measurements, and resistance mutations can be detected by sequencing of the viral genes targeted by current drugs (protease and reverse transcriptase inhibitors).

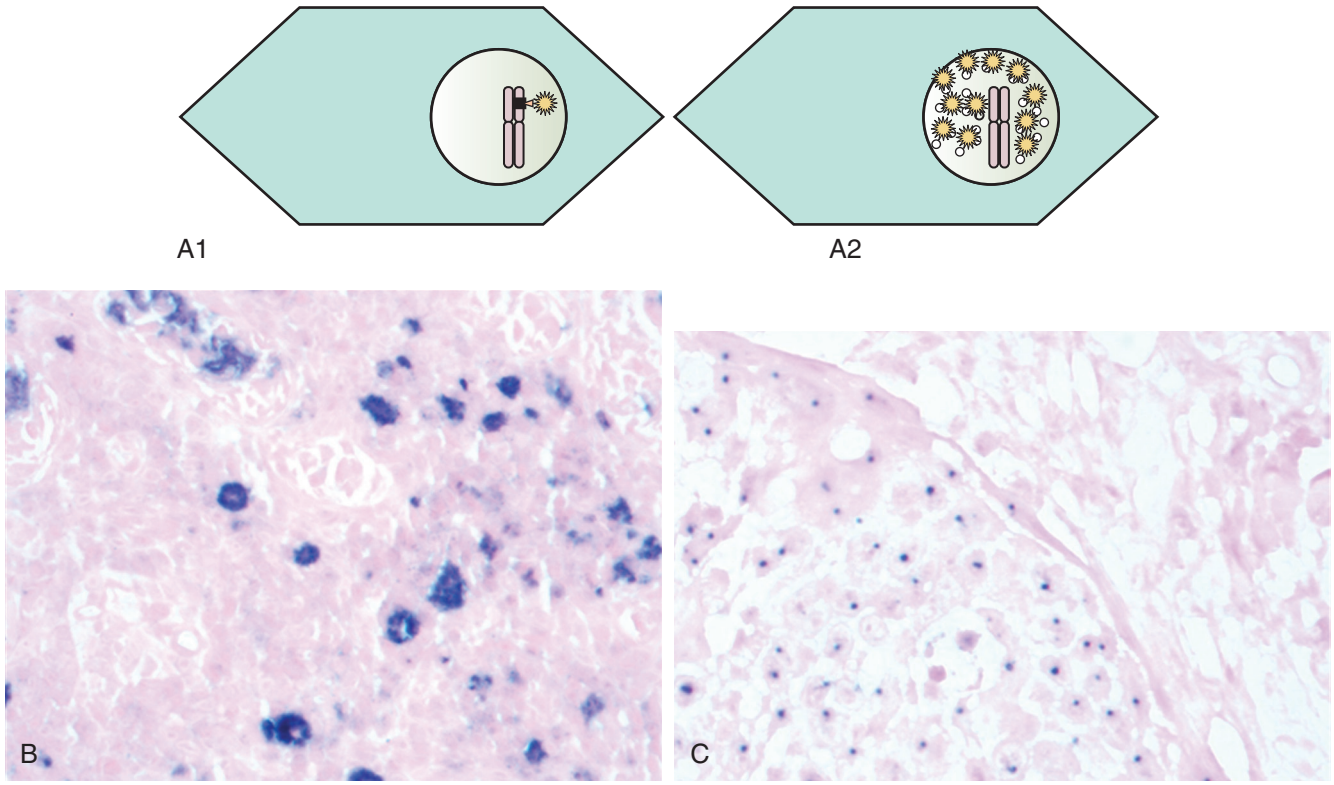
In the setting of surgical pathology a role is beginning to be developed for molecular techniques in the pathologic assessment of infection. The most relevant techniques in the setting of tissue diagnosis are in situ microbial detection using nucleic acid probes (in situ hybridization [ISH]) and PCR using nucleic acids purified from tissue sections. These two techniques can allow for diagnosis when special stains and immunohistochemical stains are insensitive (e.g., low antigen expression), and in some instances they can allow for speciation when microbes are identified with those techniques. In addition, molecular identification can accelerate definitive diagnosis with organisms that grow slowly or not at all in culture (e.g., fastidious organisms such as mycobacteria).

In Situ Hybridization

ISH is a technique that uses fluorescent or radiolabeled nucleic acid probes to recognize specific microbial sequences in tissue sections. The probes contain RNA or DNA sequences complementary to the target genetic elements and allow for specific localization of microbes in tissue or within cells. Depending on the sequence, some ISH probes can also bind specifically to nucleic acids from individual species, allowing for differentiation of organisms with variable virulence. Although there is great specificity with many ISH probes, immunohistochemistry is preferred, if possible, because of ease of incorporation into the modern automated pathology laboratory.

Most critical to the surgical pathologist is the identification of human papillomavirus (HPV) infection in cervical cancer screening, and this is most often accomplished with the use of PCR or similar techniques from liquid Pap smear specimens. ISH can also be very effective for definitive detection of high-risk HPV subtypes in cervical biopsy analysis for dysplasia and in defining HPV status of oropharyngeal squamous cell carcinomas.[15] Immunohistochemistry for HPV antigens has not proved sensitive enough (although p16 positivity is a reasonable surrogate marker of HPV infection). The HPV genome is present as episomes in low-grade lesions, so ISH reveals diffuse and intense staining (Fig. 2.22). In high-grade lesions and invasive carcinomas, the HPV genome integrates into the host genome, and ISH reveals a punctate nuclear signal.

ISH is also useful in the detection of Epstein-Barr virus (EBV) in lymphocytes, including in lymphoproliferative disorders, because the EBV-encoded RNAs (EBER1 and EBER2) are expressed at very high levels. Commercial EBER ISH assays have been developed and are automatable.



• **Figure 2.22** **A,** Human papillomavirus (HPV) infection is manifested by viral genomes present as A1 episomes or as A2 integrated DNA. Therefore in situ hybridization (ISH) probes give diffuse *(lower right)* or discrete *(lower left)* staining patterns in HPV-positive tissue. Actual ISH is shown in two oropharyngeal squamous cell carcinomas, with episomal signals **(B)** and integrated virus **(C)**. (Photographs courtesy of Dr. Jennifer Hunt, Cleveland Clinic Department of Pathology.) (×250).

In 2016, a novel chemical variant of DNA called peptide nucleic acids (PNAs), consisting of nucleoside bases joined by a peptide backbone rather than a sugar backbone, has been used to detect microbial genetic material. PNA probes offer the advantage of chemical stability and higher sensitivity and specificity. These properties offer the opportunity of developing probes that can differentiate species in situ. Such probes have proved successful in differentiating tuberculous from nontuberculous mycobacterial infections by targeting the 16S ribosomal RNA; others have detected *Staphylococcus aureus*, *Enterococcus faecalis*, and *Candida* species.[16-19]

Polymerase Chain Reaction

PCR amplification to detect infectious agents in surgical pathology specimens is now so common that a basic description of the technique is unnecessary.[20,21] PCR is without a doubt the most sensitive detection method available, and because it can be performed on archived formalin-fixed, paraffin-embedded samples, important diagnoses can be rendered even if cultures were not obtained from tissue biopsies at the time of processing (e.g., lung wedge resections for tumor that later reveal necrotizing granulomas). Common applications of PCR to surgical samples are listed in Table 2.5.

However, central to PCR is the requirement that the exact RNA or DNA sequences to be amplified must be known. Abundant microbial sequence data are publicly available, so that any equipped laboratory can analyze the most known pathogens.

TABLE 2.5 Applications of the Polymerase Chain Reaction to Microbial Detection in Tissues

Organism	Pathologic Process
Aspergillus	Invasive aspergillosis[21]
Human papillomavirus	Cervical HSIL and LSIL, HPV-positive squamous cell carcinoma of the oropharynx
Herpes virus	Herpes encephalitis (CSF or brain biopsy)
Mycobacteria	Necrotizing granulomatous inflammation
Bartonella	Cat-scratch disease or bacillary angiomatosis
Enterovirus, adenovirus, influenza A virus	Viral myocarditis[20]

CSF, Cerebrospinal fluid; *HPV*, human papillomavirus; *HSIL*, high-grade squamous intraepithelial lesion; *LSIL*, low-grade squamous intraepithelial lesion.

There are a number of well-conserved genes in microorganisms, such as the ribosomal 16S gene and the heat shock protein/chaperonin HSP60/65 (or GroEL), that are excellent targets for PCR. Analysis of the16S ribosomal RNA gene in bacteria by PCR and subsequent sequencing is especially informative because there are well-conserved sequences that can be used as binding sites for

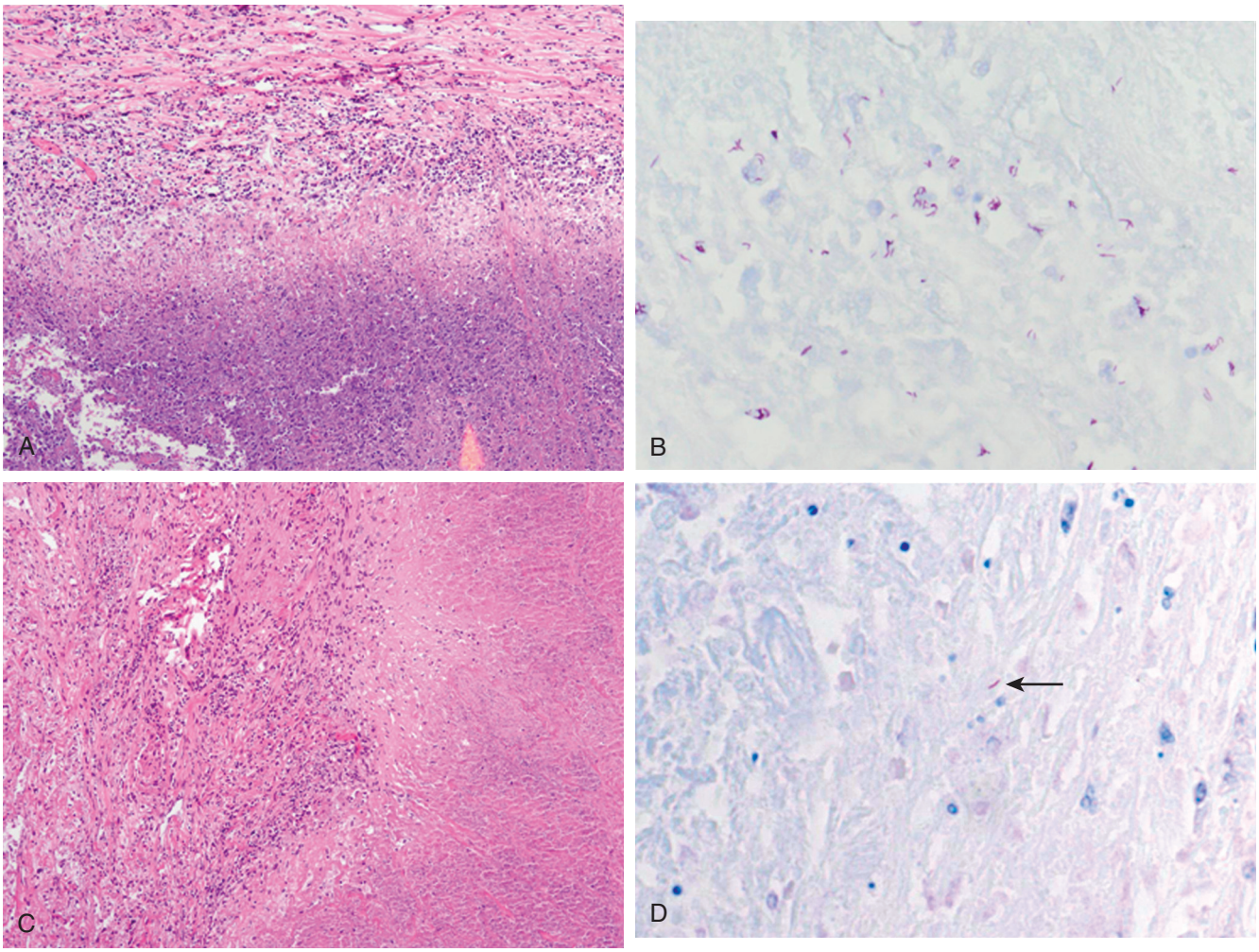
universal PCR primers adjacent to variable sequences and then analyzed and compared with databases of known sequences (Fig. 2.23).

This sensitivity of PCR is not without its problems. Many of the most important infectious agents seen in general practice are found in the environment and can contaminate surgical specimens. For example, *Aspergillus* and mycobacterial species are normal denizens of the clinical laboratory; if samples are not kept sterile, they may come in contact with these species during tissue processing. Even more vexing, such species also can cause opportunistic infections, and so their identification cannot always be dismissed as clinically irrelevant.

Branch Chain In Situ Hybridization

A branch chain in situ hybridization (bISH) assay is yet another platform for detecting infectious organisms in formalin-fixed, paraffin-embedded tissue (Fig. 2.24).[22] This technology uses a branched DNA structure for signal amplification, but, unlike PCR, the target is not amplified. The signal amplification greatly increases the sensitivity of the assay, although it is significantly lower than PCR-based techniques. Multiple tiling probes for a gene are hybridized to the target RNA, and these are typically distributed throughout the genome of interest, greatly increasing the sensitivity of the assay. A signal amplification structure is created by a series of hybridization steps. The final steps involve hybridization of multiple alkaline phosphatase–conjugated label probes, and the eventual branched structure has approximately 400 binding sites for the alkaline phosphatase–labeled probe, which can be visualized as a red chromogen with a bright field microscope. The signal is typically seen as dots, although with highly expressed transcripts individual dots are no longer visualized. These assays are commercially available and can be performed on clinical grade automated immunohistochemistry instruments.

Although it is theoretically possible to develop probes against virtually any organisms, the bISH assay is generally successful in cases in which the target is highly expressed, such as EBV, cytomegalovirus (CMV), and high-risk (HR) HPV infections. The bISH assay detects E6 and E7, transcripts that are highly expressed



• **Figure 2.23** Two cases of mycobacterial infection. **A,** *Mycobacterium avium-intracellulare* complex (MAC) infection in a lung specimen with **(B)** numerous acid-fast bacteria (AFB). **C,** Lung specimen with *Mycobacterium tuberculosis* complex (MTb) infection with rare AFB (*arrow* in **D**) (×200).

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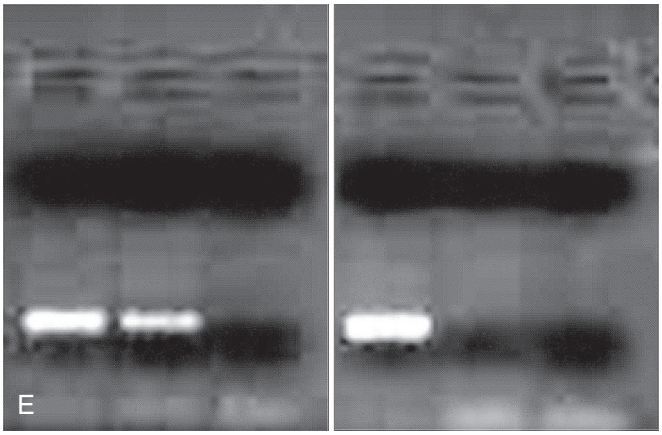

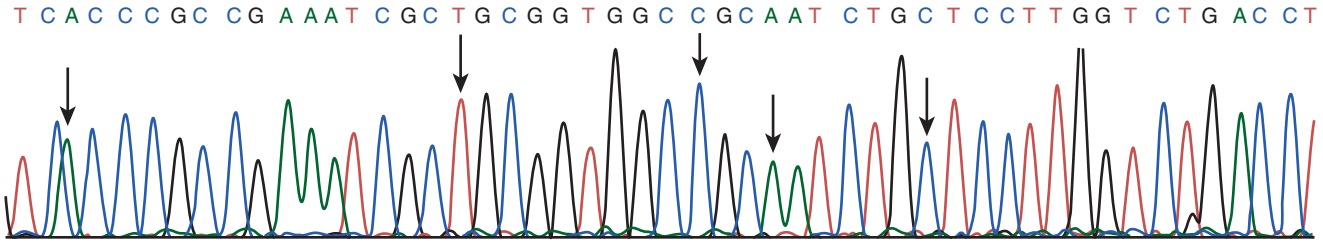

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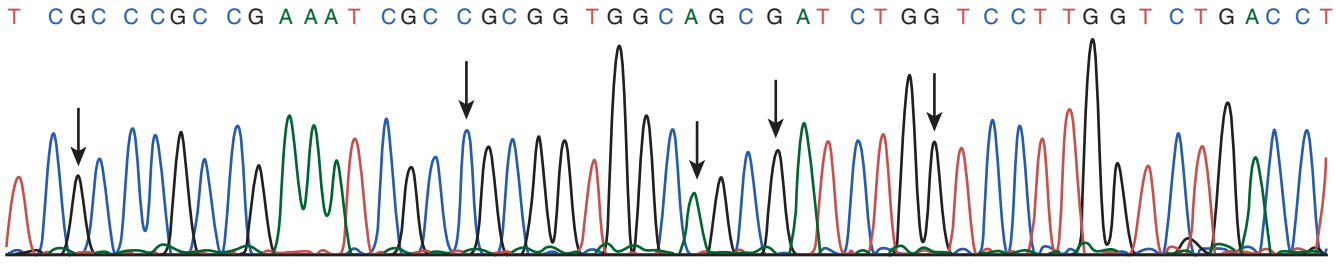

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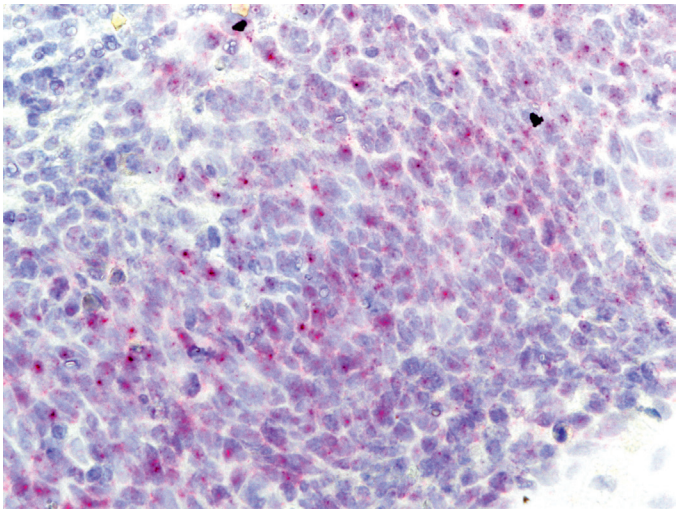
• **Figure 2.23, cont'd** DNA was isolated from formalin-fixed, paraffin-embedded tissue and amplified with primers to the *HSP65* gene, showing positive bands for the MTb sample (**E,** *lane 1*) and the MAC sample (**E,** *lane 2*). An MTb-specific gene polymerase chain reaction assay (*IS6110* gene) reveals a band with only MTb (**E,** *lane 4*). Water-only control lanes show no band in either reaction (**E,** *lanes 3 and 6*). The *HSP65* gene products were subject to DNA sequencing with the bacterial sequence from the MTb sample in **F** and from the MAC in **G**. Alignment of the two sequences reveals numerous sequence differences *(arrows)* in the region, which can be used to differentiate the two species.



• **Figure 2.24** This bISH RNA assay for high-risk HPV targets HPV-16 E6 and E7 transcripts. The red chromogen in this head and neck squamous cell carcinoma is noted within both the nucleus and the cytoplasm.

in HPV-related squamous cell carcinoma. The sensitivity of the RNA-based assay for HR HPV is higher than DNA-based assays.[23] Furthermore, the RNA assay is biologically more relevant because it detects transcripts key to oncogenic transformation.

The limitations of the bISH RNA assay need to be recognized. Nonspecific staining, although generally fewer than 1 dot per 10 cells, could be significantly higher, making it impossible to distinguish true from nonspecific signal. Poor RNA quality could undermine the assay, and rapid formalin fixation is often necessary for optimal preservation of RNA. Finally, although in theory the assay is capable of detecting a single transcript, most successful clinical-grade bISH assays express several hundred target RNA transcripts per cell.

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