9 Bronchoalveolar Lavage: Microbial Evaluation

Bacteriology, Virology, Parasitology, Mycology, and Airway Microbiome

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Introduction: Overall Diagnosis of Infection by Bronchoscopic Techniques

Flexible bronchoscopy with bronchoalveolar lavage (BAL) or intraluminal (transbronchial) biopsy can be an invaluable tool in diagnosing pulmonary infectious disease, and in many cases, it may guide treatment. Bronchoscopy allows for both visualization of airway and mucosal surfaces and sampling that can reveal local inflammatory, immunological, or pathogenic processes.

Direct visualization may reveal signs of infection (mucus production, erythema, and edema) in the trachea, mainstem bronchi, and subsegmental bronchi. While more distal airways are more difficult to visualize, especially in smaller children and infants, mucus plugging may direct the bronchoscopist to particular lobes that appear to harbor infection. Even with localized disease, mucus and edema may be present throughout the larger airways due to local immune responses and a functional mucociliary elevator.

Local sampling can be accomplished through BAL or biopsy. The retrieved specimens can then be subjected to standard techniques in microbial culture, cytology, histopathology, along with other molecular tests. Because of higher rates of adverse complications in biopsy, BAL is generally preferred as the initial diagnostic tool [1–5], but biopsy may be necessary in cases where the infection is mostly intraparenchymal, and it may have a higher diagnostic yield [5, 6]. Standard culture techniques still represent the gold standard for identifying potential pathogens, but it should be noted that overall diagnostic yield is limited with many studies reporting rates of less than 50% [7, 8]. Multiple new molecular and culture-independent approaches are being developed, which may improve this yield.

While bronchoscopy is generally considered to be a safe procedure, risks and benefits of the procedure should always be weighed, particularly in the unstable or immunocompromised patient [2, 9–11]. Bronchoscopic evaluation with sampling by either BAL or biopsy may be considered in the following specific situations: (1) critically ill patients who warrant bronchoscopy for broad microbiologic testing and rapid diagnosis; (2)

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high-risk patients (i.e., CF, immunocompromised state, history of lung transplant, and concern for or diagnosis of interstitial lung disease) with radiographic findings consistent with an infectious etiology; (3) any child with high clinical suspicion for mycobacterial or fungal disease and who is unable to expectorate sputum; and (4) children who have failed to respond, worsened, or relapsed after empiric therapy. Young children are often not able to produce sputum or cough forcefully enough to expectorate, and bronchoscopy may represent the only way to obtain diagnostic samples from the lower respiratory tract, although induced sputum can be attempted following hypertonic saline nebulization to assist with mucus production.

The nonspecific findings (e.g., nodules, tree-in-bud, or ground-glass opacities) seen on chest imaging prior to bronchoscopy often constitute the major motivation for pursuing an infectious workup with bronchoscopy, and the imaging can often help direct the bronchoscopist to a lobe of particular disease. However, imaging is not an absolute prerequisite to bronchoscopy, and in cases where imaging is not available or in cases of diffuse disease, bronchoalveolar lavage may have a higher yield in the right middle lobe and/or lingula.

The ultimate goal of bronchoscopy in the infectious disease context is to garner information that will lead to changes or refinements in therapy. Table 9.1 displays an approach to the diagnostic evaluation for infection using bronchoscopy. Despite low definitive diagnostic yields, several studies have shown that bronchoscopy has an impact on treatment decisions [5, 8, 12, 13]. In addition to identifying a pathogen that can be specifically targeted over a defined duration, the results of bronchoscopy, even if they are not completely definitive, can help simplify the antimicrobial regimen, limiting unnecessary exposure to antibiotics and all of the attendant risks such as organ toxicities, allergic reactions, microbial dysbiosis, and the fostering of antibiotic resistance.

This chapter discusses the diagnosis of infections using bronchoscopy with an emphasis on microbiology. We also discuss relevant histopathologic, immunologic, molecular, and culture-independent diagnostic techniques.

Bacteriology

Bacterial Etiologies and Sampling

The most common bacterial causes of lower respiratory tract infection (LRTI) are familiar respiratory pathogens that cause community-associated pneumonia (CAP), such as *Streptococcus pneumoniae*, *Haemophilus influenzae*, and *Moraxella catarrhalis*. However, the relative importance of each of these organisms is difficult to determine because most disease is treated empirically and most infections go uncultured because the site of infection is difficult to access. *Staphylococcus aureus* is an unusual cause of CAP (approximately 1% overall), but the severity of infection is high, with patients frequently requiring mechanical ventilation and often presenting with parapneumonic effusion [14–16]. Therefore, *S. aureus* should always be considered in serious cases of pneumonia. In addition, over the past 15 years, there has been heightened concern in the United States, based on increased rates of *S. aureus* CAP associated with the community-associated methicillin-resistant *Staphylococcus aureus* (MRSA) epidemic lineage USA300 [14], that anti-MRSA treatment may need to be considered in severe pneumonia.

Bronchoscopy with BAL or biopsy for culture is rarely performed in uncomplicated CAP, but it presents a diagnostic option for more complicated bacterial pneumonia or pneumonia that fails to respond to empiric antibiotic treatment. De Shutter et al reported high rates of nontypeable *Haemophilus influenzae* (NTHi) in BAL cultures from patients with nonresponding or recurrent CAP [17]. This study also identified *M. catarrhalis* and *S. pneumoniae* as common pathogens. Tsai et al (2017) reported "viridans" group streptococci, *Pseudomonas aeruginosa*, and *Staphylococcus aureus* as the most common pathogens in BALs from patients with nonresponding CAP. Rates of detection of a pathogen

Table 9.1 Recommended testing for bronchoscopic-based sampling in cases when infection is clinically suspected

	Cytology	Bacteria	Viruses	Fungi	Mycobacteria	Other
Routine testing (all patients)	Quantitative cell count Microscopy Consider: Oil red-O stain (for lipid-laden macrophages) Iron stain (for hemosiderin-laden macrophages) Periodic Acid-Schiff (PAS) stain	Stains: Gram stain Culture: Quantitative aerobic respiratory culture	Molecular tests: PCR-based basic viral panel (if testing not performed on NP specimen)	Stains: Grocott-Gomori methenamine silver (GMS) stain Periodic acid Schiff (PAS) stain Calcofluor white KOH Culture: Fungal culture	Stains: AFB Culture: Liquid and solid AFB culture	Consider: Mycoplasma PCR
Additional testing to be considered in immune-compromised individuals	Microscopy for parasites and fungi (particularly important when geographic exposures suggest specific infections not amenable to other testing) Pathology of biopsied samples		Pathogen-specific PCRs: EBV (in solid organ or hematopoietic cell transplant patient) CMV (in solid organ or hematopoietic cell transplant patient) VZV (when disseminated disease is suspected) HHV-6 (hematopoietic cell transplant or severe immune compromise; clinical relevance often difficult to interpret) HSV (in disseminated disease; clinical relevance may be difficult to interpret given prevalence of shedding from oral mucosa)	Molecular tests: *Pneumocystis jirovecii* PCR Other tests: *Aspergillus* galactomannan *Histoplasma* antigen (if exposure in endemic region) *Cryptococcus* antigen (rare cases)	Consider: Xpert/RIF® for TB	Consider: *Toxoplasma gondii* PCR (solid organ transplant recipients, particularly heart transplant) Legionella DFA

in nonresponding or recurring CAP have been reported to be as high as 76% when the lower respiratory tract is sampled [17].

Other diagnostic sampling techniques for LRTI have even poorer yields. When blood cultures are done in the setting of LRTI, they are positive in less than 3% of cases [18–21], although some studies have reported rates as high as 7% [22] or 11% [23] in community-acquired pneumonia (CAP). It is important to note that severity of pneumonia is positively associated with the likelihood of obtaining a positive blood culture with rates of 13–26% in the setting of an empyema or parapneumonic effusion [18, 24–27]. Sputum cultures can be difficult to obtain in younger children who cannot expectorate and may be reflective of commensal colonization with potential pathogens rather than the etiology of a LRTI [28]. Thoracentesis and transthoracic needle aspiration may have higher culture yields, but they are also significantly more invasive and are uncommon especially in less severe disease [28].

The so-called "atypical" causes of LRTI such as *Mycoplasma pneumoniae* and *Chlamydophila pneumoniae* are more common in children >5 years [29–31]. These bacteria can be detected in BAL fluid [32, 33], but swabs of the upper respiratory tract (nasopharyngeal, oropharyngeal) as well as throat and nasal washes are often used to rule out LRTI [28]. If children can produce sputum, it may be the preferred sample for diagnosis of these organisms [28].

Legionella pneumophila is also a cause of LRTI that has been associated with infections both in hospitals and in the community [34]. *L. pneumophila* can be detected from BAL fluid using culture, which requires specific media (BYCE) and can take 3–5 days to grow, or by polymerase chain reaction (PCR)-based techniques, which produce results much more quickly but with less sensitivity [35, 36]. In practice, most disease is diagnosed by the urine antigen test, a monoclonal antibody test that specifically targets *L. pneumophila* serogroup 1. Because serogroup 1 causes anywhere from 50% to 80% of Legionnaire's disease, it is possible that cases are missed when this single modality is used [35].

Staphylococcus aureus, *Pseudomonas aeruginosa*, and *Haemophilus influenzae* are the most prevalent causes of LRTI in ventilator-associated pneumonia (VAP); other common causes include gram-negative pathogens such as *Klebsiella* spp., *Enterobacter* spp., *Escherichia coli*, *Serratia* spp., and occasionally *Acinetobacter* spp. [37–39]. The role of anaerobic bacteria in VAP is not well understood, but it is likely that there are high levels of exposure and possibly colonization with commensal anaerobes during intubation and ventilation [40]. The gold standard for diagnosis of VAP is direct observation of the infected tissue and culture, and thus requires bronchoscopy [41]. However, the diagnosis is often made through clinical and radiographic findings because of the risks associated with more invasive procedures [42, 43]. Comparison between different methods for obtaining cultures in VAP, including BAL, nonbronchoscopic (NB) BAL, transbronchial biopsy, tracheal aspiration (TA), protected specimen brush, and postmortem autopsy, has shown enormous heterogeneity and incongruence [37, 42–53]. BAL is a generally accepted reference sampling method, but in clinical practice, TA is more often performed because of feasibility and safety [48, 54, 55]. However, TA is likely to be contaminated with upper respiratory microbiota or may simply represent colonization of the endotracheal tube, and therefore, it has low specificity [49, 52, 56].

Common bacterial causes of LRTI in immunocompromised hosts encompass both the common causes of CAP (e.g., *S. pneumoniae* and *H. influenzae*) and more opportunistic pathogens associated with VAP (*P. aeruginosa* and *S. aureus*) [57, 58]. Despite the appropriate emphasis on diagnosis of fungi and some viruses in these patients, potentially pathogenic bacteria are identified in about a third of positive BAL samples [8, 57]. It is, however, important to reiterate that overall yields in immunocompromised pediatric patients vary widely from 28% to 68% [8].

Bronchoscopy with BAL is often used in cystic fibrosis (CF) to determine the presence of LRTI with common bacterial CF pathogens such as *P. aeruginosa*, *S. aureus*, *H. influenzae*,

Stenotrophomonas maltophilia, *Achromobacter xylosoxidans*, *Burkholderia cepacia* complex as well as nontuberculous mycobacteria (NTM) and fungi, which will be discussed below. Anaerobic bacteria such as *Prevotella* spp. have recently been appreciated to be important microorganisms in CF, though their role as pathogens is not firmly established [59–61]. In CF, the prevalence of pathogens is linked to the age of the patient, with *S. aureus* and *H. influenzae* being more prevalent in young children, and gram negatives such as *P. aeruginosa* becoming dominant in the adolescent years [62]. In addition, the distribution of pathogens throughout the lung can be variable with both phenotypic variation within a species [63–66] (e.g., antibiotic resistance, metabolic status, and mucoidy), as well as species composition [67–69] (e.g., relative abundance, presence/absence). Noninvasive upper respiratory tract sampling (oropharyngeal, nasopharyngeal, and cough swabs) and expectorated sputum are commonly used for surveillance of pathogens in CF, and studies have reported conflicting negative and positive predictive values for LRTI compared to BAL [70–77]. One study showed that in adults, BAL and protected brushing samples were not superior to sputum samples in *P. aeruginosa* yield and may have given a more representative picture of the entire infecting population [78]. Other studies showed no clinical or cost benefit, and higher rates of adverse events with treatment based on BAL culture (reviewed in [79]). BAL may be especially useful in children who cannot expectorate [70, 80, 81], but induced sputum (using hypertonic saline) is an emerging alternative that appears to be safe and has potentially higher microbiological yields than other noninvasive sampling techniques [82–86]. Indeed, some studies have shown that induced sputum may have the same or better microbiological yield than BAL [87–90]. Although there is considerable doubt about the overall utility of BAL in CF microbiological diagnosis, it remains the gold standard for pathogen detection at the site of infection, and may be useful on a case-by-case basis, or if there are unexplained clinical changes without changes in surveillance microbiology.

Methods for Detecting Bacterial Infection in Bronchoscopic Samples

Culture

After gram staining for standard bacterial pathogens, which can give an initial diagnostic indication of the possible, but clearly not definitive, disease etiology [91], BAL fluid is subjected to standard bacterial cultures. While there is heterogeneity in clinical laboratory practice, most laboratories use an array of different culture media (e.g., blood, chocolate, and MacConkey and colistin–nalidixic acid agars) with additional media used for specific diseases (e.g., *B. cepacia* media used in CF cultures). While certain organisms may be reported by laboratories at any abundance (e.g., MRSA, *Nocardia* spp., *P. aeruginosa*), quantitative culture techniques have been shown to enhance the interpretation of BAL culture [28, 92–95]. A cutoff of $>10^3$ colony forming units (CFUs) has been shown to be significant with sensitivity of 90% and specificity of 97% [28, 96], but many studies and laboratories use $>10^4$ CFUs, which yield specificities as high as 100% [94, 97, 98]. The standard respiratory pathogens such as *S. pneumoniae*, *H. influenzae*, and *M. catarrhalis* as well as common bacterial VAP and CF pathogens have relatively high yields with standard culture, though published yields vary greatly [28]. *M. pneumoniae* and *C. pneumoniae* are rarely cultured in clinical practice because of their fastidious growth requirements. As noted above, culture may be helpful for detection of *Legionella pneumophila*, but this requires special culture techniques and a longer culture time [35]. It is also worthwhile noting that samples are rarely grown anaerobically, and therefore will not detect obligate anaerobes.

One important limitation of culture is that it relies on the retrieval of living bacteria. Indeed, prior exposure to antibiotics has been shown to affect accuracy and yields [7, 45, 99–101]. One study reported that yields were 63% when the BAL was done within 3 days of starting treatment, whereas this dropped to 58% between 3

and 14 days after starting treatment and 34% after 14 days of treatment [7].

PCR/Nucleic Acids

Culture remains the primary diagnostic modality for most bacterial causes of LRTI, but PCR-based techniques may increase yield especially after the initiation of antibiotics [102]. Targeted PCR-based techniques are available for *S. pneumoniae* [103–108], and these tests are easiest to interpret when used on pleural fluid samples, for which one study showed to have 78% sensitivity and 93% specificity [103]. Directed PCR-based strategies have been developed for *H. influenzae*, *S. aureus,* and *M. catarrhalis* as well [109–112]. Only a few studies address targeted PCR for respiratory pathogens in BAL [113, 114], but new multiplex PCR systems may offer high sensitivity and specificity [115]. The diagnostic utility of PCR for *S. pneumoniae* in other samples such as sputum and throat swabs is unclear, and they are plagued by the problem of differentiating between colonization and infection [112, 116–119], a problem that might be partially remedied by considering absolute bacterial burden [107, 120–122]. Conflicting results have been seen with PCR for *S. pneumoniae* blood and serum samples [116, 120, 123–126]. The test for *S. pneumoniae* urine antigen may be a more sensitive and cost effective [127]. Broad range 16S rRNA amplicon approaches that can detect multiple pathogens form pleural fluid have only marginally better yields compared to culture [128].

PCR and nucleic acid techniques are favored for detection of *M. pneumoniae* and *C. pneumoniae*, and they are often included in PCR respiratory viral panels. As noted above, these are often obtained from upper respiratory tract samples of the nasopharynx or oropharynx. However, the available data suggest that yields may be better from sputum [28] probably due to the overall abundance of the organism in the sample. There are only very limited available data on the yields from BAL specimens. PCR-based tests are commercially available for detection of *L. pneumophila*, but they have limited sensitivity [35].

Mycobacteriology

Mycobacteria are gram-positive, aerobic, acid-fast bacilli that cause significant disease worldwide, primarily affecting vulnerable populations [129–133]. Categorization of these organisms is classically defined by the Runyon classification, which relies on observation of phenotypic traits (growth rate and photochromogenicity) [134]. Further, mycobacteria may be defined by their parasitism (facultative versus obligate) and ecologic predilection (saprophytic, zoonotic, and human pathogens). For clinical purposes, these organisms are most deductively classified by the disease as they manifest in humans: *Mycobacterium tuberculosis* complex (i.e., *M. tuberculosis*, *M. bovis*, *M. africanum*) causing pulmonary or extrapulmonary tuberculosis; *Mycobacterium leprae* causing leprosy; and nontuberculosis mycobacteria (NTM) causing pulmonary, disseminated, and soft-tissue infections. In this subsection, we focus on pulmonary manifestations of tuberculous and nontuberculous disease with regard to diagnostic challenges, the role of bronchoscopy, and available microbiologic diagnostic techniques.

Tuberculosis

As the oldest documented human pathogen, tuberculosis (TB) remains the leading cause of death by an infectious agent worldwide with an estimated 1.7 billion people infected and nearly two million deaths per year [135]. Multidrug resistant TB (MDR-TB) harboring resistance to two of the first-line antimicrobial agents (isoniazid and rifampin) affects over 500,000 new patients per year [135]. The World Health Organization and United Nations have developed Sustainable Development Goals (SDGs) as part of the End TB Strategy by 2035, which is threatened mostly by increasing drug resistance and sociopolitical challenges [135]. Despite these global initiatives, socioeconomic factors (e.g., extreme poverty, overcrowding, malnutrition, and living in a developing country) are still the major determinants of clinical outcomes [136].

TB does not have an environmental or zoonotic reservoir and is instead transmitted from person-to-person by the inhalation of droplet nuclei (1–5 μm) filled with acid-fast bacilli that have been expelled into the air by a patient with active TB. While exposure does not imply infection, over 95% of infected individuals will advance to latent TB infection (LTBI), a state of clinical quiescence and slow bacterial replication held at bay by an intact adaptive immune system. Progressive primary tuberculosis is rare and occurs in patients with deficiencies in adaptive immunity, young children, and the elderly. However, most infections in children and adolescents are asymptomatic. Reactivated TB, prompted by the development of a risk factor (e.g., solid organ transplant, immunosuppression, HIV/AIDS), classically presents with productive cough, fever, weight loss, growth delay, chills, and night sweats. Active pulmonary TB in adults is distinguished by cavitary lung disease with caseous, necrotizing granulomas favoring the upper lobes. However, children rarely have cavitary disease, but rather present with nonspecific radiographic findings (atelectasis, pleural infiltration or effusion, mediastinal lymphadenopathy, lower lung abnormalities, or a military pattern). Congenital TB presents with a sepsis-like picture, bronchopneumonia, and hepatosplenomegaly. In all ages, TB disease can span the entire airway, including laryngeal, tracheal, and endobronchial involvement [137, 138].

Diagnosis of active pulmonary TB requires positive delayed-type hypersensitivity (DTH) reaction by positive tuberculin skin test (TST) with respiratory symptoms and radiographic evidence of disease. In the absence active TB, LTBI is diagnosed by positive TST or interferon-γ (INF-γ) assay (IGRA). Traditionally, diagnosis of active disease is confirmed by three positive acid-fast bacilli (AFB) cultures at least 8 hours apart [138]. Childhood tuberculosis can be challenging diagnostically because of their inability to produce sufficient sputum and the wide range of possible radiographic findings and presentations. The Red Book currently recommends all children with suspicion for TB who cannot produce sputum to have three consecutive early morning gastric aspirates for AFB smear and culture [138]. While not established as the first line of specimen collection in children, obtaining a radiographic-directed BAL sample for smear and culture can be extremely useful diagnostically. Furthermore, as TB affects the larger airways in children with rates of 41–63%, endobronchial disease can be directly observed, classified, and sampled via bronchoscopy [139]. In addition, endobronchial disease can result in severe airway obstruction or strictures that require bronchoscopic intervention (Fig. 9.1).

Treatment should be guided by antimicrobial susceptibility testing and directed by an infectious disease specialist.

Nontuberculous Mycobacteria

NTMs are present ubiquitously in the environment, living in biofilms in water and soil, and cause opportunistic disease in at-risk populations [140–145]. Immunocompromised hosts, the elderly, patients with cystic fibrosis (CF), chronic obstructive pulmonary disease (COPD), interstitial lung disease, and non-CF bronchiectasis are at increased risk due to decreased mucociliary clearance, inflamed or bronchiectatic airways, and structural lung damage. Susceptibility is also increased in patients with immune deficiencies affecting INF-γ, interlukin-12 (IL-12), and tumor necrosis factor-alpha (TNF-α) signaling pathways, as in the case of HIV infection or treatment with a TNF-α inhibitor [146]. Patients with pulmonary alveolar proteinosis (PAP) are at higher risk for NTM infection, as well [146].

In the United States, the largest studies estimate prevalence in the general population at about 5.3 per 100,000 persons with highest rates in elderly over 80 years old and those living in southeastern states and in Hawaii [129, 130, 132, 147, 148]. Increases in prevalence are estimated to be rising at rates of 2.6–11.8% per year [130–132, 148, 149]. A global collaboration headed by the NTM-Network European Trials Group (NTM-NET) reviewed over 20,000 patient samples from 30 countries and 6 continents and identified *Mycobacterium avium* complex (MAC) as

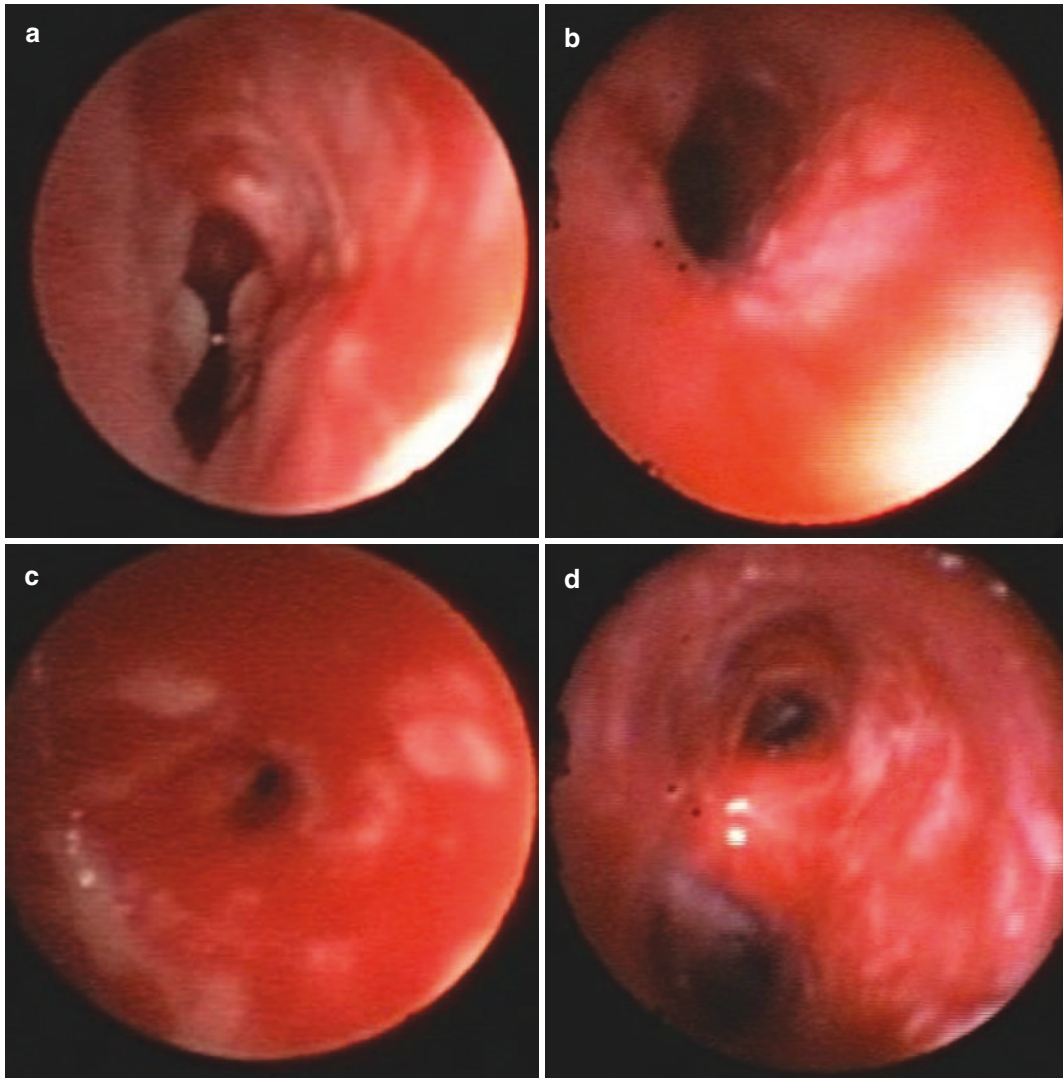

Fig. 9.1 Endobronchial TB causing stenosis of the left mainstem bronchus (**a**) and left upper lobe bronchus (**b**) in a 12-year-old boy with active TB. Postballoon dilation, narrowing is improved in (**c**) and (**d**), respectively

the predominant species complex worldwide at 47%, followed by *M. gordonae* (11%), *M. xenopi* (8%), *M. fortuitum* complex (7%), *M. kansasii* (4%), and *M. abscessus* (3%) [150]. *M. gordonae*, *M. terrae*, and *M. fortuitum* complex are often environmental contaminants and are unlikely to cause disease [151, 152]. The major causes of pulmonary disease in humans include MAC, *Mycobacterium abscessus* complex (MABSC), and *Mycobacterium kansasii* [130, 143, 146, 153–157].

Transmission typically occurs due to environmental exposure by inhalation route of aerosolized mycobacteria. Clinical manifestations of pulmonary NTM infection range from silent, chronic colonization to severe, progressive lung disease. The American Thoracic Society (ATS) and Infectious Disease Society of America (IDSA) 2007 diagnostic criteria require pulmonary symptoms, specific radiographic findings (nodular or cavitary opacities or multifocal bronchiectasis) with exclusion of other diagnoses, and

microbiologic evidence of disease by either: two positive AFB cultures or one positive culture from bronchial washing, bronchoalveolar lavage (BAL), transbronchial biopsy (TBB), or endobronchial ultrasound-guided (EBUS) biopsy with histopathologic features consistent with mycobacterial disease [151].

In the CF population, average NTM prevalence in the United States has climbed from 1.3% in 1984 to an average of 14% in 2014, with some states as high as 28% [129, 147, 154, 157–159]. Possible causes for the rise of recognized NTM disease in CF are manyfold and include longer patient life-expectancy, development of NTM-adapted niches due to use of broad-spectrum antibiotic usage, and increased awareness and testing as per the 2013 Cystic Fibrosis Foundation (CFF) update on infection prevention and control guidelines [160]. Risk factors for NTM disease in CF are widely debated. The largest cross-sectional studies of CF patients to date suggest associations between NTM disease and better lung function, higher rates of coinfection with *Staphylococcus aureus* and lower rates of coinfection with *Pseudomonas aeruginosa*, a history of allergic bronchopulmonary aspergillosis (ABPA) or coinfection with *Aspergillus fumigatus*, and the chronic use of azithromycin or systemic steroids. However, smaller, less robust studies have not successfully replicated all of these associations and in some cases have demonstrated contradictory results [156, 157, 161–167]. Universally in CF, increased age is the most predictive risk factor for acquisition of NTM infection, which is likely secondary to repeated and prolonged exposure to the pathogen as well as host factors [140, 145, 166, 167]. Environmental studies have shown increased prevalence of NTM in areas associated with higher levels of atmospheric water and closer living proximity to water, although these associations tend to be region-specific [129, 156, 157, 162, 164–166, 168–178]. Widely debated is the potential for human transmissibility, which has been reported in the literature [176, 179] and is generally accepted to be a possible albeit rare modality for transmission.

Diagnosis of NTM in CF is problematic because (1) lung disease caused by mycobacterial infection resembles findings of the chronic progression of severe CF lung disease (tree-in-bud nodularity, bronchiectasis, and cavitation), and (2) NTM disease manifestations range from silent, chronic colonization to severe, progressive lung disease [161, 166, 167, 180–182]. In 2016, the Cystic Fibrosis Foundation (CFF) and European Cystic Fibrosis Society (ECFS) published a statement to assist clinicians with NTM diagnosis and treatment in CF [183]. The guidelines agree with the ATS/IDSA statement and additionally recommend chest high-resolution computed tomography (HRCT) to characterize disease and guide BAL sampling when indicated. When diagnostic criteria are met and clinical decline is appreciated despite standard CF care, treatment should be pursued and managed by an infectious disease specialist.

Similar to TB, antimicrobial regimens for NTM should be guided by susceptibility testing. Initial therapies for susceptible MAC pulmonary infection include a macrolide (clarithromycin or azithromycin), ethambutol, and either rifampin or rifabutin. Severe or cavitary MAC disease may warrant initiation with an IV aminoglycoside (amikacin or streptomycin). Typically, susceptible MABSC requires a more aggressive approach including initiation with IV amikacin, IV cefoxitin, IV imipenem or meropenem, and clarithromycin. For both complexes, other antimicrobial agents (fluoroquinolones, doxycycline or minocycline, linezolid, clofazimine, cycloserine, ethionamide, and capreomycin) or novel therapies (inhaled GM-CSF) are sometimes necessary [184]. Despite susceptibility-guided, multidrug regimens, NTM often acquires antibiotic resistance and is unable to be eradicated [151, 155, 159, 183, 185, 186]

Methods for Detecting Mycobacterial Infection in Bronchoscopic Samples

Many children are not able to produce sputum at a sufficient quantity for mycobacterial microbiologic testing, thus bronchoscopy with bronchoalveolar lavage can be an indispensable component in the

diagnosis of mycobacterial disease. In the critically ill patient, bronchoscopy for BAL, transbronchial biopsy (TBB), or endobronchial ultrasound-guided (EBUS) biopsy may be the only way to identify the etiology of pulmonary infiltrates or endobronchial disease. Furthermore, patients with cystic fibrosis and suspicion for NTM disease who are smear negative by sputum should undergo HRCT-guided bronchoscopic sampling as recommended by the CFF/ECFS [183].

Culture

The "gold standard" for diagnosis of mycobacterial infection is the AFB culture. The ATS/IDSA and CFF/ECFS recommend that both solid (Lowenstein–Jenson or Middlebrook 7H11) and liquid culture (Middlebrook 7H9) techniques be performed following standard decontamination measures (0.5% N-acetyl L-cysteine, 2% NaOH). Due to increased sensitivity and more rapid detection, liquid culture is recommended to be performed by the BD BACTEC™ MGIT™ Automated Mycobacterial Detection System which utilizes Middlebrook 7H9 liquid broth supplemented with 0.2% glycerol, 10% OADC (Oleic Albumin Dextrose Catalase), and PANTA antibiotic mixture (polymyxin B, amphotericin B, nalidixic acid, trimethoprim, azlocillin). Growth in liquid culture is faster than solid culture, and thus, positivity may be revealed sooner. For the clinician to be comfortable with negative results, both liquid and solid cultures must be finalized, with solid cultures requiring up to 8–12 weeks for appreciable growth to occur.

Molecular-Based Testing

All mycobacteria isolated by culture should be identified to the species level, primarily to distinguish between TB and NTM disease and secondarily because species classification dictates both treatment and anticipated outcomes [152, 187]. Current molecular techniques include nucleic acid amplification tests (NAAT) such as polymerase chain reaction restriction fragment length polymorphism (PCR-RFLP) analysis, real-time PCR (RT-PCR), and line probe assays (LPA), chemiluminescent DNA probes, DNA sequencing, and matrix-assisted laser desorption ionization-time of flight spectrometry (MALDI-TOF) [138, 152, 188, 189]. Mycobacterial gene targets for NAAT include *rpoB* [190, 191], *hsp65* [192, 193], 16S rRNA genes [194–196], the 16S–23S gene spacer [197, 198], and *groES* [199]. Many clinical microbiology laboratories utilize MALDI-TOF for species identification due to its ability to speciate mycobacteria (63.8–98.6%) at a low cost [200–204]. Despite the advent of new technologies, few of the above modalities are able to classify organisms to the subspecies level, as is possible with whole-genome sequencing (WGS). As sequencing costs continue to decline, WGS will likely be utilized more in basic clinical diagnosis of mycobacterial infection.

For suspicion of TB disease, rapid NAATs can be useful for culture-independent diagnosis, though a negative test does not exclude disease and cannot replace standard culture. The first molecular test endorsed by the World Health Organization (WHO) in 2010 was the Xpert® MTB/RIF (Cepheid, USA), an assay that employs RT-PCR of the *rpoB* gene [135, 190]. By a large review (Cochrane review with expansion per the WHO 2013 Updated Report), the Xpert® MTB/RIF assay had a pooled sensitivity of 88% and pooled specificity of 99% for all specimens tested (expectorated and induced sputum, BAL, tissue samples, gastric aspirates, nasopharyngeal aspirates, and extrapulmonary samples) [205]. In children, sensitivities were 55–90%, 40–100%, and 40–100% for expectorated sputum, induced sputum, and gastric lavage, respectively, with specificities for all sites between 93% and 100%. One study has evaluated the efficacy of this test on BAL fluid in children with suspected TB and found 53% sensitivity and 100% specificity [206].

Pathology/Cytology

Staining and direct microscopy should always accompany AFB culture. The current recommendation for AFB staining is the fluorochrome tech-

nique, though Ziehl–Neelsen (ZN) and auramine–rhodamine (AR) staining methods may also be employed [151]. In the case of *M. tuberculosis*, and as is generalized to NTM pulmonary disease, smear positivity is associated with increased infectivity, higher bacterial loads, and worse disease burden. However, AFB smears can be negative in close to 50% of culture-positive patients [207]. During active TB infection, BAL cell counts will reveal a lymphocytic alveolitis with "foamy" (AFB-laden) macrophages and may have high percentages of immature macrophages (monocytes) thought to influx from the blood [137]. Biopsy specimens taken during bronchoscopy or by wedge resection will show granulomatous inflammation and should also be directly stained to identify AFB [146].

Virology

Introduction

Viruses are the most frequent cause of upper and lower respiratory tract infections in pediatric patients [208]. The challenges in diagnosing viruses as the cause of pneumonia are several-fold: (a) some viruses demonstrate prolonged shedding from the oropharynx or upper respiratory tract and detection in the upper respiratory tract may not reflect active lower respiratory tract infection [209, 210], (b) culture- and molecular-based detection methods do not distinguish infection from shedding or colonization [211], and (c) bacterial–viral and viral–viral coinfections are common [208]. Bronchoscopic approaches may help clinicians identify viral pathogens but do not necessarily solve the issue of distinguishing infection from shedding.

Respiratory Viruses

Several viruses fall into a group commonly referred to as "respiratory viruses." These viruses are from different families and have varying pathogenicity, but all have a predilection for causing respiratory tract infections. The most common viruses in this group are respiratory syncytial virus (RSV), influenza A and B, parainfluenza 1–3, human metapneumovirus (hMPV), adenovirus, human coronavirus (HCoV), and rhinovirus [208, 212], although numerous other viruses have been associated with pneumonia in children. Respiratory viruses are all more common in younger children [208, 213–215], likely due to a combination of social factors/exposures and immune naivety to these pathogens. Viral respiratory tract infections are typically self-limited, but can be life threatening in infants [216], immunocompromised children [217, 218], and children with underlying medical conditions such as asthma, heart disease, or cystic fibrosis [219].

The frequency of viral–bacterial and viral–viral coinfections makes estimation of the incidence of viral LRTIs due to specific pathogens challenging. In a recent study of 2219 children hospitalized with community-acquired pneumonia (CAP) at one of three US hospitals, viruses were detected in two-thirds [208]. Coinfections were present in 26% of all children with CAP, including 19% that had multiple viruses detected [208]. Thus, while studies report the incidence of detection of respiratory viruses in children with pneumonia, the proportion of pediatric pneumonia specifically caused by each organism is not known.

Viral pneumonia from respiratory viruses almost always develops as a result of progression from a preceding upper respiratory tract infection. Therefore, the diagnosis of viral LRTIs in children generally occurs via molecular detection of virus (i.e., PCR) or viral antigens in nasopharyngeal (NP) samples [208, 215]. Detection of these pathogens in lower respiratory tract specimens via bronchoscopy can represent pneumonia, but may also occur in cases of viral shedding, colonization, or contamination from upper respiratory tract secretions. In general, there is good concordance between PCR testing from nasopharyngeal swab and BAL samples for the detection of respiratory viruses [220, 221], limiting the need for more invasive procedures such as bronchoscopy. In fact, studies in children have demonstrated higher yield for detection of respiratory viruses from NP samples compared with BAL [222], although this may reflect the location of

viral replication among various viral pathogens. Therefore, bronchoscopic procedures are reserved for those children with negative testing from upper respiratory tract samples or for whom other nonviral processes are being considered.

Herpesviruses

Herpesviruses are common viral infections that establish life-long latency in human hosts. Herpes simplex virus (HSV), cytomegalovirus (CMV), human herpesvirus-6 and -7 (HHV-6 and HHV-7), Epstein–Barr virus (EBV), and varicella zoster virus (VZV) all have capacity to cause lower respiratory tract infections in the setting of primary infection or reactivation, particularly in severely immunocompromised individuals [223–226]. However, viral shedding is common in both immunocompromised and nonimmunocompromised individuals, and detection of these viruses in the respiratory tract does not confirm disease [209, 210]. Therefore, clinicians utilize bronchoscopic and BAL findings, along with imaging characteristics, to establish herpesviruses as the cause of pulmonary symptoms [223].

Cytomegalovirus

Cytomegalovirus is the most common herpesvirus to cause LRTI and is associated with significant morbidity and mortality in immunocompromised children, most notably solid organ transplant (SOT) and hematopoietic stem cell transplant (HSCT) recipients [223], as well as patients with HIV/AIDS [224, 225]. Detection of CMV in the blood is common in these patients [227, 228] and does not necessarily indicate CMV disease. Histopathology has been the gold standard for the diagnosis of tissue-invasive CMV disease, demonstrating characteristic nuclear inclusions on biopsied tissue samples [211]. Immunohistochemistry can supplement histopathology, staining for CMV antigens in infected tissue cells and facilitating identification of nuclear enlargement and intranuclear inclusions [211, 229]. Although culture of a lower respiratory tract sample has good specificity for CMV pneumonia, culture does not distinguish between viral shedding and invasive respiratory tract disease [211]. Thus, histopathologic evidence of end-organ damage is preferred for definitive diagnosis of CMV pneumonitis/pneumonia [211].

Polymerase chain reaction (PCR) testing has replaced the use of culture for detection of CMV in clinical specimens. The exquisite sensitivity and negative predictive value of PCR from BAL samples make it a reliable test for ruling out CMV pneumonia [230]. In the correct clinical context, detection of CMV from BAL specimens supports the diagnosis of CMV pneumonia or pneumonitis. Yet, as with culture, mere detection of CMV by PCR cannot distinguish infection from viral shedding in the respiratory tract. Quantification of CMV viral load in BAL specimens, however, may facilitate the distinction between infection and viral shedding [231]. Unlike in blood, higher viral loads correlate with findings on immunohistochemistry staining from lung biopsy samples [229]. CMV quantification from BAL specimens has been predictive of CMV pneumonitis in lung transplant [232, 233] and HSCT recipients [231], as well as in infants [234]. In a seminal study from Boeckh et al., a viral load of 500 IU/mL reliably differentiated CMV pneumonia from asymptomatic shedding in adult HSCT patients [231]. However, the specific viral load associated with CMV pneumonia in pediatric populations has not been established and should not be assumed to be the same as that found in adults. Although the optimal cut-off to distinguish pulmonary infection from respiratory tract shedding varies across studies and patient populations [231–235], quantification of CMV viral load is more specific for CMV infection than detection of CMV by qualitative PCR from respiratory tract specimens.

Herpes Simplex Virus

The incidence of HSV pneumonia in children is not well known. Hypoxemia is the most striking clinical feature of HSV pneumonia, which can be profound [236]. Patients at highest risk include transplant recipients and other immunocompromised patients [236–238], most often from reactivation of latent infection, and mechanically

ventilated patients, who may develop disease as a result of inoculation of contaminated oral secretions [236]. As with CMV, detection of HSV from respiratory tract samples is not diagnostic. In critically ill adults, HSV has been detected by PCR of BAL samples from 30% to 50% of patients [239–241]; the presence of HSV in the respiratory tract of critically ill adults most often represents viral shedding during reactivation and not invasive infection [241]. Histopathology can detect characteristic viral inclusions in cases of HSV pneumonia [236], when biopsies are performed, and more reliably distinguishes tissue-invasive infection from shedding than PCR. The clinical significance of detection of HSV in critically ill children, who have a much lower seroprevalence of HSV than adults, is not known.

In neonates with disseminated HSV, pneumonia may be present in up to 50% of cases [242]. While pneumonia can rarely be the presenting feature [243], the diagnosis of disseminated neonatal infection is made via detection of virus in the blood in combination with systemic symptoms. Bronchoscopy with BAL provides limited added information in these cases and should never delay the initiation of antiviral therapy.

Varicella Zoster Virus

Varicella zoster virus (VZV) can cause severe, life-threatening pneumonia, most often in adults, pregnant women, and immunocompromised individuals [244, 245]. Varicella pneumonia develops almost exclusively in the context of disseminated infection, and tracheal and bronchial ulcers can be visualized on bronchoscopy shortly after the development of skin rash [244, 246]. Because varicella pneumonia is a complication of disseminated disease, and skin lesions are generally present, PCR from skin lesions or blood is likely to diagnose the majority of cases. Respiratory tract specimens are rarely required.

Other Herpesviruses

Epstein–Barr virus, as well as HHV-6 and HHV-7, can be detected on lower respiratory tract samples among immunocompromised and critically ill patients in the setting of viral reactivation [209, 247]. Because the respiratory tract is a common site of EBV latency, viral DNA can be detected in up to 50% of both immune-competent and immune-compromised patients. Given the frequency of EBV, HHV-6, and HHV-7 detection in BAL specimens in both immunocompromised and immunocompetent hosts, the role of these viruses in causing or contributing to lower respiratory tract disease is unknown.

EBV is the causative agent of posttransplant lymphoproliferative disorder (PTLD), which can affect any organ system including the airway. Laryngoscopy and/or bronchoscopy with biopsies can help identify EBV-positive B cells within affected tissues that are characteristic of PTLD [248–250]. Although the airway is a rare site of PTLD, endoscopic procedures may be necessary to confirm the diagnosis.

Human Papillomavirus (HPV)

There are more than 60 serotypes of HPV, which vary in their propensity for human infections. Certain serotypes of HPV can cause recurrent respiratory papillomatosis, a disease consisting of the development of persistent or recurrent epithelial nodules in the airway, most commonly affecting young children and young adults [251]. Clinical symptoms consistent with airway irritation (cough, hoarseness, and voice change) or obstruction (stridor and respiratory distress) may be suggestive of this process. But, definitive diagnosis is made via direct visualization of the lesions via laryngoscopy and/or bronchoscopy [251]; biopsies demonstrate the characteristic papillomas. Medical treatment options are limited (cryotherapy, laser therapy, and intralesional therapies) and surgical approaches may be needed to alleviate obstruction and more debilitating symptoms.

Histopathology/Direct Microscopy

Histopathology is the traditional technique for confirmation of tissue-invasive viral infection. Direct microscopy of respiratory tract specimens is of minimal utility for diagnosing viral infections because viruses cannot be visualized by tra-

ditional microscopic techniques. Histopathology and immunohistochemical staining methods facilitate identification of infiltration of tissues by viral pathogens, including respiratory viruses and herpesviruses [215], by demonstrating characteristic patterns of cellular damage. Respiratory viruses are most often associated with diffuse alveolar damage or interstitial pneumonia [252]; more severe cases may cause necrotizing bronchitis and intra-alveolar hemorrhage. Meanwhile, herpesviruses and adenoviruses cause necrotizing bronchiolitis, as well as the formation of characteristic intranuclear or intracytoplasmic inclusions, which are collections of nucleoproteins and virions [252, 253]. Because the patterns of injury are nonspecific, immunohistochemistry (IHC) or in situ hybridization (ISH) techniques are used to confirm the presence of specific viruses within cells using virus-specific antibodies [252]. Measles virus is characterized by intranuclear and intracytoplasmic eosinophilic inclusions and the presence of multinucleated giant cells [252].

Culture

Viral culture techniques have been utilized traditionally to detect the presence of viruses in clinical samples, including respiratory secretions. Tube culture, which facilitates detection of cytopathic effects in infected cells, and shell-vial culture, which utilizes immunofluorescent techniques to detect viral growth [211, 254], are the approaches most often used for detection of viral pathogens. Shell-vial culture is much faster than tube culture, taking 1–2 days instead of weeks [211]. But, viral cultures are being replaced clinically by the use of molecular detection methods, such as PCR, which are much more sensitive, specific, and cost-effective, and significantly less time-consuming. Because many viruses infect upper airways, while others shed from the oral mucosa or upper respiratory tract, cultures from transbronchial biopsies (i.e., tissue cultures) are more suggestive of viral infection than those performed on BAL fluid.

PCR/Nucleic Acid Testing

Nucleic acid amplification, most often via PCR, has become the diagnostic modality of choice for most viral infections. Multiplex PCR panels can detect the presence of numerous viruses in respiratory tract samples, such as nasopharyngeal (NP) swabs, NP aspirates, induced sputum, or BAL fluid [255]. PCR testing is severalfold more sensitive than culture- and antigen-based methods for detecting viral pathogens in respiratory samples [215]. There is good concordance between PCR testing from NP swabs and BAL samples for the detection of respiratory viruses [220, 221], making NP samples the preferred diagnostic specimens in children when these common viral pathogens are being considered. Because of the potential for prolonged viral shedding following an infection, and because viruses can colonize airways, as well, detection of viral DNA by PCR needs to be interpreted in the appropriate clinical and epidemiologic contexts to support a diagnosis of viral pneumonia.

Antigen-Based Testing

Immunofluorescent techniques (immunochromatographic testing) or enzyme immunoassay (EIA) tests can rapidly detect viral antigens in respiratory specimens, most often NP samples. Test results are available in minutes, making them highly valuable point-of-care tests. Rapid antigen testing for influenza and RSV (target: RSV fusion surface protein) are commercially available and the most common rapid viral tests used in children [256]. Antigen detection is influenced by the viral load present in the sample, so rapid antigen detection tests tend to be less sensitive than PCR [215, 257]. A 2015 meta-analysis by Chartrand and colleagues reported a pooled sensitivity and specificity of RSV rapid antigen tests of 80% (95% CI: 76–83%) and 97% (95% CI: 96–98%), respectively [257]. These authors performed a separate meta-analysis evaluating

the performance of rapid influenza testing [258], reporting a pooled sensitivity of 62.3% (95% CI: 57.9–66.6%) and specificity of 98.2% (95% CI: 97.5–98.7%). Thus, rapid antigen tests perform well for ruling in RSV and influenza infections, but less well for ruling them out.

Mycology

Introduction

Infection of the respiratory tract is the most common form of invasive fungal disease (IFD) in children. Yeasts, molds, and dimorphic fungi (organisms that can grow as either a yeast or mold) are ubiquitous in the environment and cause infection of the paranasal sinuses and/or lungs following inhalation of fungal spores [259], although fungi can also disseminate hematogenously, leading to secondary pulmonary infections. Immunecompromised individuals and those with impaired airway clearance, such as with cystic fibrosis, are most prone to pulmonary IFD.

In order to facilitate the use of consistent terminology in clinical and epidemiologic research, consensus guidelines from the European Organization for Research and Treatment of Cancer/Invasive Fungal Infections Cooperative Group (EORTC) and the National Institute of Allergy and Infectious Diseases Mycoses Study Group (MSG) categorize the diagnosis of IFD into proven (Fig. 9.2), probable (Fig. 9.3), or possible cases [260]. Proven cases require histologic evidence or positive microbiologic culture from sterile site body fluids or tissue specimens [260]. This does *not* include BAL fluid or sputum. Meanwhile, the diagnosis of probable or possible IFD, which are terms used only in immunecompromised individuals, requires a combination of host factors and clinical features with (for probable) or without (for possible) mycological evidence of infection [260]. The EORTC/MSG definitions are commonly employed in research; however, it is important to recognize that they are generally not employed in clinical practice. Failure to meet these definitions does not exclude a diagnosis of IFD, and the definitions have variable sensitivity and specificity compared to histopathology in children [261]. So, while these terms promote the use of consistent terminology in the research setting, they should not be employed clinically or relied upon to guide treatment decisions. They nevertheless form the basis for many studies referred to in the following sections.

Analysis and specimen	Molds[a]	Yeasts[a]
Microscopic analysis: sterile material	Histopathologic, cytopathologic, or direct microscopic examination[b] of a specimen obtained by needle aspiration or biopsy in which hyphae or melanized yeast-like forms are seen accompanied by evidence of associated tissue damage	Histopathologic, cytopathologic, or direct microscopic examination[b] of a specimen obtained by needle aspiration or biopsy from a normally sterile site (other than mucous membranes) showing yeast cells–for example, *Cryptococcus* species indicated by encapsulated budding yeasts or Candida species showing pseudohyphae or true hyphae[c]
Culture		
Sterile material	Recovery of a mold or "black yeast" by culture of a specimen obtained by a sterile procedure from a normally sterile and clinically or radiologically abnormal site consistent with an infectious disease process, excluding bronchoalveolar lavage fluid, a cranial sinus cavity specimen, and urine	Recovery of a yeast by culture of a sample obtained by a sterile procedure (including a freshly placed [<24 h ago] drain) from a normally sterile site showing a clinical or radiological abnormality consistent with an infectious disease process
Blood	Blood culture that yields a mold[d] (e.g., *Fusarium* species) in the context of a compatible infectious disease process	Blood culture that yields yeast (e.g., *Cryptococcus or Candida* species) or yeast-like fungi (e.g., *Trichosporon* species)
Serological analysis: CSF	Not applicable	Cryptococcal antigen in CSF indicates disseminated cryptococcosis

[a] If culture is available, append the identification at the genus or species level from the culture results.
[b] Tissue and cells submitted for histopathologic or cytopathologic studies should be stained by Grocott-Gomorri methenamine sliver stain or by periodic acid Schiff stain, to faciliate inspection of fungal structures. Whenever possible, wet mounts of specimens from foci related to invasive fungal disease should be stained with a fluorescent dye (e.g., calcofluor or blankophor),
[c] *Candida, Trichosporon, and yeast-like Geotrichum species and Blastoschizomyces capitatus* may also from pseudohyphase or true hyphae.
[d] Recovery of *Aspergillus* species from blood cultures invariably represents contamination.

Fig. 9.2 Criteria for proven invasive fungal disease except for endemic mycoses. (Reprinted with permission from De Pauw et al. [260]. © 2008 by the Infectious Diseases Society of America)

Host factors[a]
- Recent histroy of neutropenia (<0.5 x 10^9 neutrophils/L [<500 neutrophils/mm^3] for > 10 days) temporally related to the onset pf fungal disease
- Receipt of an allogeneic stem cell transplant
- Prolonged use of corticosteriods (excluding among patients with allergic bronchopulmonary aspergillosis) at a mean minimum dose of 0.3 mg/kg/day of prednisone equivalent for >3 weeks
- Treatment with other recongined T cell immunosuppressants, such as cyclosporine, TNF-α blockers, specific monoclonal antibodies (such as alemutuzumab), or nucleoside analogues during the past 90 days
- Inherited severe immunodeficiency (such as chronic granulomatous disease or severe combined immunodeficiency)

Clinical criteria[b]
- Lower respiratory tract fungl disease[c]
  - The presence of 1 of the following 3 signs on CT:
    - Dense, well-circumscribed lesions(s) with or without a halo sign
    - Air-crescent sign
    - Cavity
- Tracheobronchitis
  - Tracheobronchial ulceration, nodule, pseudomembrane, plaque, or eschar seen on bronchoscopic analysis
- Sinonasal infection
  - Imaging showing sinusitis plus at least 1 of the following 3 signs:
    - Acute localized pain (including pain radiating to the eye)
    - Nasal ulcer with black eschar
    - Extension from the paranasal sinus across bony barriers, including into the orbit
- CNS infection
  - 1 of the following 2 signs:
    - Focal lesions on imaging
    - Meningeal enhancement on MRI or CT
- Disseminated candidiasis[d]
  - At least 1 of the following 2 entities after an episode of candidemia within the previous 2 weeks:
    - Small, target-like abscesses (bull's-eye lesions) in lover or spleen
    - Progressive retinal exudates on ophthalmologic examination

Mycological criteria
- Direct test (cytology, direct microscopy, or culture)
  - Mold in sputum, bronchoalveolar lavage fluid, bronchial brush, or sinus aspirate samples, indicated by 1 of the following:
    - Presence of fungal elements indicating a mold
    - Recovery by culture of a mold (e.g., *Aspergillus, Fusarium,* Zygomycetes, or Scedosporium species)
- Indirect tests (detection of antigen or cell-wall consistuents)[e]
  - Aspergillosis
    - Galactomannan antigen detected in plasma, serum, bronchoalveolar lavage fluid, or CSF
  - Invasive fungal disease other than cryptococcosis and zygomycoses
    - β-D-glucan detected in serum

NOTE. Probable IFD requires the presence of a host factor, a clinical criterion, and a mycological criterion, Cases that meer the criteria for a host factor and a clinical criterion but for which mycological criteria are absent are considered possible IFD.

[a] Host factors are not synonymous with risk factors and are characteristics by which individuals predisposed to invasive fungal disease can be recognized. They are intended primarily to apply to patients given treatment for malignant disesase and to recipients of allogeneic hematopoietic system cell and solid-organ transplants, These host factors are also applicable to patients who receive corticosteroids and other T cell suppressants as well as to patients with primary immunodeficiencies.

[b] Must be consistent with the mycological findings, if any and must be temporally related to current episode.

[c] Every reasonable attempt should be made to exclude an alternative etiology.

[d] The presence of signs and symptoms consistent with sepsis syndrome indicates acute disseminated diseses, wherease their absence denotes chronic disseminated disease.

[e] These tests are primary applicable to aspergilosis and candidasis and are not useful in diagnosing infections due to *Cryptococcus* species or Zygomycetes (e.g., *Rhizopus, Mucor, or Absidia* species). Detection of nucleic acid is not included, because there are as yet no validated or standardized methods.

Fig. 9.3 Criteria for probable invasive fungal disease except for endemic mycoses. (Reprinted with permission from De Pauw et al. [260]. © 2008 by the Infectious Diseases Society of America)

Fungi That Cause Pulmonary Infections

Aspergillus

Aspergillus species cause a myriad of clinical pulmonary presentations ranging from asymptomatic colonization to ABPA and invasive pulmonary aspergillosis (IPA). Following inhalation, *Aspergillus* species colonize the upper and lower airways. Colonization of a preexisting pulmonary cavity may lead to the formation of a fungus ball, which could remain asymptomatic for prolonged periods or cause symptoms such as cough or hemoptysis. ABPA is an allergic response to *Aspergillus fumigatus* antigens that primarily affects patients with asthma or cystic fibrosis [262, 263]. Characterized by recurrent episodes of wheezing, cough, transient pulmonary opacities, and bronchiectasis, the diagnostic criteria for ABPA are based on cutaneous hypersensitivity to *A. fumigatus* antigens, serum IgE levels (>1000 IU/mL), and two out of three of the following: presence of precipitating or IgG antibodies against *A. fumigatus* in serum, radiographic pulmonary opacities consistent with ABPA, and a total eosinophil count >500 cells/μL [264].

Invasive pulmonary aspergillosis is the most common manifestation of invasive aspergillosis (IA) and is associated with significant morbidity and mortality [265, 266]. IPA may manifest as nodular pulmonary infiltrates, pleural-based infiltrates, or cavitary lesions, and dissemination secondary to vascular invasion also occurs. Tracheobronchitis is a relatively rare form of IA that most often affects lung transplant recipients and severely immunocompromised individuals [267]. Infiltration of the bronchial or tracheal mucosa leads to ulceration, necrosis, and/or the formation of pseudomembranes, which can be visualized on bronchoscopy and confirmed by biopsy and culture [267].

The diagnosis of aspergillosis is based on clinical signs and symptoms, including imaging findings, combined with diagnostic testing. *Aspergillus* species do not grow efficiently in culture and can be morphologically similar to other molds on histologic examination [268]. Thus, confirmation of aspergillosis can be challenging. Culture remains the gold standard for confirmation of IPA, but the use of biomarkers (serum or BAL galactomannan; see below) and molecular testing (PCR, florescent in situ hybridization [FISH]) can support its diagnosis and provide more rapid information than culture.

Pneumocystis jirovecii

Pneumocystis jirovecii is a leading cause of opportunistic infection in immunocompromised patients worldwide [269]. *Pneumocystis jirovecii* pneumonia (PJP) is a rare diagnosis in children but remains an important consideration in neonates and immune-compromised or immune-suppressed pediatric populations [270]. Airway colonization is transient early in life: in a longitudinal study of 46 mother–infant pairs, 91% of infants had *Pneumocystis* identified by PCR from nasopharyngeal swabs within the first 6 months of life [271]. While colonization precedes the development of infection, it is unclear how often colonization progresses to infection versus infection which develops following new acquisition.

Clinical symptoms of PJP are highly variable and often nonspecific. In patients with HIV, PJP typically presents with subacute symptoms consisting of nonproductive cough, low-grade fever, and progressive dyspnea [269, 272], while symptoms can be more acute in non-HIV patients. Hypoxia is the most frequent sign of PJP, and the magnitude of the alveolar-arterial oxygen gradient often signals the severity of disease [269]. Chest radiographs are normal early, but frequently show symmetric, perihilar interstitial infiltrates later in the course [273]. Figure 9.4 displays the characteristic chest x-ray of an 8-month-old boy with PJP. Chest CT is the imaging modality of choice, most often showing patchy, ground-glass infiltrates [269, 273].

Pneumocystis cannot be grown in standard culture; therefore, diagnosis relies on direct microscopy, histologic evaluation, or DNA detection. Silver stain (Grocott-Gomori methenamine

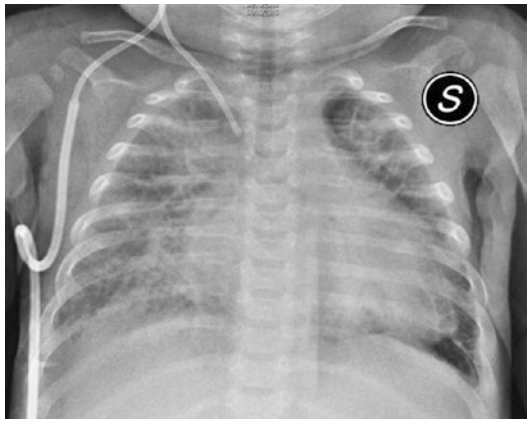

Fig. 9.4 *Pneumocystis jirovecii* pneumonia in an 8-month-old boy after bone marrow transplantation. Chest radiograph demonstrates bilateral interstitial infiltrates and patchy consolidation on the left side (S). (Reprinted with permission from Toma et al. [273]. © Springer-Verlag Berlin Heidelberg 2016)

silver stain) is the conventional method for identifying organisms, but other staining methods are also useful [269, 272]. Immunofluorescent techniques have been used to identify *Pneumocystis* [269, 274], but are labor intensive and do not outperform direct microscopy [275]. PCR from BAL is becoming the test of choice for PJP as numerous studies have demonstrated excellent sensitivity [276, 277]. (1 → 3)-β-D-glucan (BDG) is a cell wall component of many fungi, including *Pneumocystis.* BDG detection in serum or plasma has excellent sensitivity (94.8%) and good specificity (86.3%) in the diagnosis of PJP [278], but its specificity from BAL samples is low.

Candida

Candida lung disease most often develops secondary to hematogenous dissemination. This form of candidiasis typically has a diffuse, nodular pattern on imaging, consistent with hematogenous spread of infection [279]. Meanwhile, *Candida* species rarely cause primary LRTI. Colonization of the airways occurs quickly following endotracheal intubation [280]. Therefore, *Candida* are frequently recovered on culture from tracheal aspirate or BAL samples of critically ill patients, but seldom are the cause of pneumonia [281, 282]. Similarly, *Candida* are commonly recovered from sputum samples in patients with cystic fibrosis [283], yet the precise role of *Candida* in the lung disease of these patients has not been established.

Cryptococcus

Cryptococcus neoformans and *Cryptococcus gattii* are budding yeasts found in soil worldwide and are particularly important pathogens in immunocompromised hosts, most notably those with HIV. Cryptococcal lung disease occurs in both immune-compromised and immune-competent individuals [284, 285], but is rare in children. In a population-based study of cryptococcosis in the United States from 2000 to 2007, the rate of hospitalization in children was less than 0.25 per 100,000 population [286]; the specific rate of respiratory cryptococcosis was not reported. At Beijing Children's Hospital, there were 53 children hospitalized with cryptococcal disease from 2002 to 2014, half of whom had pulmonary involvement [287].

Cryptococcal species can be grown readily on bacterial or fungal culture media, and isolation from respiratory tract samples may support a diagnosis of cryptococcosis since these fungi are not typical respiratory tract flora [288]. Latex agglutination and EIA tests are available that can detect the capsular polysaccharide of both *C. neoformans* and *C. gattii* [289]. Cryptococcal antigen testing from serum and cerebrospinal fluid is sensitive for the detection of cryptococcal meningitis and disseminated cryptococcosis, but has lower sensitivity in non-CNS infections [290, 291]. Cryptococcal antigen testing from BAL fluid has limited sensitivity and positive predictive value, which does not support its use clinically [292–294].

Mucorales Species

Fungi of the subphylum Mucormycotina, the vast majority of which are in the order Mucorales, are ubiquitous, filamentous fungi found in soil and

decaying matter throughout the world [295]. Invasive infections, known as mucormycosis (formerly zygomycosis), are predominantly acquired through inhalation, but also can develop following direct inoculation of skin or mucosal surfaces [295]. Pulmonary mucormycosis develops most often as a result of inhalation of fungal spores, but can occur as an extension of sinus disease or secondary to disseminated disease. Clinically, pulmonary mucormycosis is similar to other mold infections, such as aspergillosis. Due to angioinvasive nature of Mucorales, dissemination is common (>50%), although blood cultures are rarely positive.

Diagnosis of pulmonary mucormycosis is challenging. Abnormal chest imaging in the correct host (neutropenia, transplant recipient, and diabetes with ketoacidosis) should key clinicians to the possibility of this infection. The imaging findings are nonspecific, varying from discrete solitary nodules to larger areas of confluent infection to cavitary lesions with pulmonary effusion [296]. Bronchoalveolar lavage is an important diagnostic tool in high-risk patients with imaging findings consistent with mucormycosis, facilitating identification by histopathology and culture. Mucorales have broad, thin-walled, irregular, pauci-septate hyphae with wide-angled (90°) branching on microscopic examination, which distinguishes them from other molds, such as *Aspergillus* or *Fusarium* species [275, 296]. Definitive diagnosis is made by culture, although the sensitivity of culture is poor [296]. Molecular tests, such as PCR, are not routinely clinically available for Mucorales species, and antigen tests, such as β-D-glucan and galactomannan, are not clinically useful.

Dimorphic Fungi

Dimorphic fungi are comprised of a group of fungi that can exist in either yeast or mycelial (mold) forms, depending on temperature and environmental conditions: *Blastomyces* species (*B. dermatitidis, B. gilchristii*), *Coccidioides* species (*C. immitis*, *C. posadasii*), *Histoplasma capsulatum*, *Paracoccidioides* species (*P. brasiliensis*, *P. lutzii*), *Sporothrix schenckii*, and *Talaromyces marneffei* (formerly *Penicillium marneffei*). The mold phase allows these organisms to survive in the environment, while the yeast phase promotes virulence, immune evasion, and development of human infections [297, 298]. These organisms, often referred to as endemic mycoses, are geographically limited and found in specific ecologic niches within their endemic areas. Histoplasmosis, coccidioidomycosis, and blastomycosis are the most likely to manifest as pulmonary infections [298]. In the United States, histoplasmosis occurs predominantly in the Midwest and Southeast, as does blastomycosis, while coccidioidomycosis occurs in the Southwest [299]. Paracoccidioidomycosis is a cause of CAP in Central and South America [300].

Pulmonary infection by endemic mycoses follows inhalation of aerosolized mycelial forms of the fungi. The majority of infections are self-limited, but more severe manifestations, including death, can occur in the setting of a large inoculum of infection or in an immune-compromised individual [301, 302]. Acute pulmonary infections typically present as focal, consolidative processes, similar to CAP in their symptomatology and radiographic appearance [297, 301, 303]. Additional nonspecific symptoms such as fatigue, arthralgias/myalgias, and chills commonly accompany this stage of infection. Mediastinal and hilar adenopathy are often seen on radiographs in patients with acute histoplasmosis and to a lesser degree in patients with coccidioidomycosis [304].

For all endemic mycoses, the definite diagnosis is made by identifying fungi on histopathology, cytopathology, or culture. Histoplasmosis is associated with the formation of caseating and noncaseating granulomas [302, 305]. Serologic tests (complement fixation, immunodiffusion) are available to aid in the diagnosis of blastomycosis, coccidioidomycosis, and histoplasmosis, but have variable sensitivity depending on the form of disease and duration of infection. Histoplasma antigen testing by EIA can be performed on urine, blood, and BAL fluid, but it is positive not only in cases of histoplasmosis but

also infections caused by *Blastomyces, Paracoccidioides,* and *Talaromyces marneffei* [306]. Antigenuria and antigenemia are more often detected in cases of disseminated infection than in patients with isolated pulmonary infections [304, 307].

Methods to Diagnose Fungal Pulmonary Infections

Histopathology/Direct Microscopy

Direct microscopy of BAL or lung tissue specimens is often the first test performed when pulmonary fungal infection is suspected [275]. Although less sensitive than culture, positive direct microscopy is helpful since growth of fungi in culture can take days to weeks. Many fungi can be identified based on their morphologic characteristics (Table 9.2), although not to the species level [289]. Use of 10–20% potassium hydroxide (KOH) facilitates identification of fungi by degrading proteins within specimens with the exception of fungal cell walls, promoting visualization of hyphae and conidia [275]. The addition of other stains, such as Calcofluor white, can further augment identification of fungi. The most sensitive stain used to identify fungi in tissue and BAL fluid specimens is Grocott-Gomori methenamine silver (GMS), which stains almost all fungal cell walls [259]. GMS (aka "silver") stain is the conventional method for identifying *Pneumocystis* organisms from sputum or BAL fluid samples [269, 272], as well as Mucorales, *Aspergillus*, and other fungi that cause invasive disease [275, 296]. Periodic acid Schiff (PAS) stains are also useful for detecting fungal hyphae [289].

Culture

Culture is the primary method for diagnosing pulmonary IFD, supporting speciation of organisms and antimicrobial susceptibility testing, when possible [289]. Culture is more sensitive than direct microscopy [275] and should be routinely performed on all BAL specimens when fungal infection is considered. Special fungal media are often utilized, which contain antibiotics to inhibit growth of bacteria. Several fungi, particularly molds, do not readily grow in culture and yield may be as low as 30–50% even when visualized by histologic and cytologic examination [308]. *Pneumocystis jirovecii* cannot be grown in routine culture; therefore, diagnosis relies on direct microscopy, histologic evaluation, or DNA detection in respiratory samples [269].

Bronchoalveolar lavage is an important diagnostic tool in patients at high risk for pulmonary IFD. Because the pathogenesis of primary pulmonary IFD involves inhalation of fungal spores, colonization of the airway necessarily precedes infection. Therefore, culture in itself is insufficient to establish the diagnosis of pulmonary IFD with any fungal organism. Culture must be combined with the clinical features (symptoms, imaging findings) and host factors to make a definitive diagnosis [260].

PCR/Nucleic Acid Testing

PCR testing is clinically available for select fungi: *Candida, Aspergillus,* and *Pneumocystis jirovecii.* Distinguishing between colonization and invasive infection is a major limitation of use of PCR from BAL for each of these fungi, however. In the appropriate clinical context, identification of *Aspergillus* or *Pneumocystis* by PCR from BAL fluid increases the posttest probability of invasive infection and may assist clinicians when other diagnostic tests (i.e., culture) are negative.

PCR has become a valuable tool in the diagnosis of invasive aspergillosis (IA), most often when performed on serum or whole-blood samples. Unfortunately, data in pediatric patients are limited. A 2016 meta-analysis in pediatric cancer and HSCT patients reported a pooled diagnostic performance of PCR for screening of IA: specificity 43–85%, sensitivity 11–80%, positive predictive value (PPV) 20–50%, and negative predictive value (NPV) 60–96% [309]. Meanwhile, the performance of PCR for the

Table 9.2 Diagnostic features of fungi associated with respiratory tract infections

Organism	Microscopic characteristics
Yeasts	
Cryptococcus spp. (*C. neoformans, C. gattii*)	Spherical budding yeasts of variable size, 2–15 μm in diameter. Capsule may be present or absent. No hyphae or pseudohyphae. Stain red with Mayer's mucicarmine stain; India ink stain used for cerebrospinal fluid samples
Pneumocystis jirovecii	Cysts are round, collapsed, or crescent shaped. Trophozoites seen on staining with Giemsa, methenamine silver (GMS), or immunofluorescent stains
Trichosporon	Hyaline arthroconidia, blastoconidia, and pseudohyphae, 2–4 by 8 μm
Molds	
Hyaline hyphomycetes (*Aspergillus, Acremonium, Fusarium, Paecilomyces, Phialemonium, Scedosporium, Scopulariopsis, Trichoderma*)	Hyaline, septate dichotomously branching (45° angle) hyphae of uniform width (3–6 μm)
Mucorales (*Absidia, Cunninghamella, Mucor, Rhizomucor, Rhizopus, Saksenaea*)	Broad, thin-walled, pauci-septate hyphae, 6–25 μm wide with nonparallel sides and random branches (90° angle)
Dematiaceous hyphomycetes (*Alternaria, Bipolaris, Curvularia, Cladophialophora, Dactylaria, Exophiala, Phialophora, Ramichloridium, Wangiella)*	Pigmented (brown, tan, or black), septate hyphae, 2–6 μm wide
Dimorphic fungi	
Blastomyces dermatitidis	Large (8–15 μm diameter) thick-walled budding yeast cells. The junction between mother and daughter cells is typically broad-based. Cells may appear multinucleate. Occasionally stains red with Mayer's mucicarmine stain
Coccidioides spp. (*C. immitis, C. posadasii*)	Spherical, thick-walled spherules, 20–30 μm diameter. Mature spherules contain small, 2–5 μm diameter endospores. Released endospores may be mistaken for yeast. Arthroconidia and hyphae may form in cavitary lesions
Histoplasma capsulatum	Small (2–4 μm diameter), intracellular, budding yeasts. Associated with caseating granulomas
Paracoccidioides brasiliensis	Large (2–30 μm diameter), multiple-budding yeasts. 12 or more narrow neck buds of variable size may arise from the mother cell, daughter yeasts may be in a "pilot-wheel configuration"
Penicillium marneffei	Oval, intracellular yeast cells bisected with a septum (fission yeast)
Sporothrix schenckii	Elongated or "cigar-shaped" yeast cells of varying size (rare). Tissue reaction forms asteroid bodies

Adapted with permission from Lease and Alexander [275] © Thieme Medical Publishers, 2011

diagnosis of IA during febrile periods was highly variable across studies: specificity 36–83%, sensitivity 0–100%, PPV 0–71%, and NPV 88–100% [309]. The sensitivity of *Aspergillus* PCR is negatively affected by the administration of antifungal therapy [310], which is relevant considering that many patients with suspected IPA are receiving antifungal prophylaxis at the time.

In patients undergoing bronchoscopy, pathogen-specific PCR testing may be valuable. In a systematic review by Avni, the diagnostic performance of PCR in BAL fluid for diagnosing proven/probable IPA was similar to that of galactomannan (sensitivity 82–86%, specificity 95%), while the sensitivity of either test being positive increased to 97% (95% CI 83–99.5) [311]. Numerous studies have reported excellent performance of PCR from BAL samples for the diagnosis of *Pneumocystis* pneumonia – pooled sensitivity of 98% and specificity of 91–93% – making it a highly useful test [21, 22]. Although several authors have suggested that quantitative

PCR can help differentiate active infection from colonization [23–25], the fungal load varies among different patient populations (i.e., HIV- vs non-HIV-infected patients, adults vs children) and threshold values that distinguish infection from colonization have not been clearly established, especially in children.

Panfungal PCR, which uses primers targeting the internal transcribed spacer (ITS) 1 and/or 2 region, can identify the presence of fungi within clinical samples. This approach is particularly useful in instances when fungi are visualized microscopically within specimens, but cultures are nondiagnostic. Because of the presence of colonizing flora within respiratory tract samples, panfungal PCR may have higher accuracy from tissue specimens, as opposed to BAL samples [312]. Next-generation sequencing also has the potential to identify fungi within culture-negative specimens, but it is best served from a sterile-site rather than respiratory tract samples.

Antigen-Based Testing

Histoplasma capsulatum antigen can be detected by EIA in serum, urine, and BAL fluid specimens in patients with histoplasmosis [305]. Antigen detection is both a rapid and sensitive adjunctive testing method for the diagnosis of histoplasmosis, although cross-reactivity occurs with other endemic mycoses including *Blastomyces*, *Paracoccidioides,* and *Talaromyces marneffei* [306]. Urine antigen detection is more sensitive in disseminated histoplasmosis than in primary pulmonary infection. In a multicenter study of patients with histoplasmosis, antigen was detected in the urine of 145 of 158 (91.8%) patients with disseminated histoplasmosis, but only 19 of 50 (38.0%) cases with acute or subacute pulmonary infections [307]. Antigen detection from BAL is more sensitive than blood or urine testing in patients with pulmonary histoplasmosis. In a study by Hage et al. that included 31 patients with pulmonary histoplasmosis, the diagnostic performance of *Histoplasma* antigen detection in BAL fluid for the diagnosis of histoplasmosis was as follows: sensitivity 93.5%, specificity 97.8%, PPV 69.1%, and NPV 99.7% [313].

Several commercially available latex agglutination and EIA tests have been developed for detection of cryptococcal polysaccharide capsule antigen, both of *C. neoformans* and *C. gattii* [289]. These tests are predominantly performed in serum and cerebrospinal fluid samples. The sensitivity of serum cryptococcal antigen is higher in individuals with disseminated and central nervous system (CNS) infection than in those with isolated lung disease [290, 291]. In a report of HIV-negative adult patients with cryptococcal disease, only 56% of 71 patients with pulmonary disease had a positive serum cryptococcal antigen test compared to 87% of those with CNS infection [290]. Cryptococcal antigen testing from BAL fluid has demonstrated variable sensitivity (71–100%) and poor positive predictive value (36–67%) in adults [292–294]. The performance of cryptococcal antigen on BAL fluid in children is unknown, but its usefulness is likely narrow considering the rarity of cryptococcal pneumonia in children.

Galactomannan

Galactomannan (GM) is a cell-wall component of *Aspergillus* species [289]. Detection of GM in serum or BAL fluid samples using ELISA is an indirect test that can support the diagnosis of IPA [260]. While GM is generally specific to aspergillosis, it can also be detected in serum of patients with penicilliosis [314]. Additionally, false-positive results have been reported in patients treated with aminopenicillin/β-lactamase combination agents [315].

In adults, serum and BAL GM correlate significantly in patients with IPA [316, 317]. An optical density index of ≥0.5 from either serum or BAL fluid is most often used as the cut-off for a diagnosis of IPA. However, BAL GM has been reported to have a higher sensitivity but lower specificity than serum GM [318–321]; therefore, some authors have suggested using a higher GM cut point from BAL fluid should to limit false-positive results [321, 322].

Data on GM from BAL fluid in pediatric patients are limited. de Mol et al. retrospectively evaluated the performance of GM from BAL fluid among 41 cases of proven/probable IA and found that a GM ≥0.5 had a sensitivity, specificity,

PPV, and NPV of 82%, 88%, 82%, and 87%, respectively [316]. Similarly, Mohammadi and colleagues found that a BAL fluid GM ≥0.5 had a sensitivity and positive predictive value of 87.5% and 93.3% [323]. Meanwhile, Desai et al. reported that a cut-off value of ≥0.5 had a sensitivity for proven/probable IA of 78% and a specificity of 84% among their pediatric cohort, while a cut-off value of 0.87 had sensitivity of 78% and specificity of 100% among a subset of immunocompromised children [317]. Additional studies are needed to establish the optimal cut-off of GM from BAL fluid in pediatric patients, but GM appears to be a valuable adjunctive test to support a diagnosis of IPA in children.

β-D-glucan

(1 → 3)-β-D-glucan (BDG) is a cell wall component of many fungi and is considered an indirect test of probable IFD with these fungi [260]. BDG is a cell component of *Candida*, *P. jirovecii*, *Aspergillus*, *Fusarium* species, *Trichosporon, Coccidioides, Histoplasma*, and others, but not of *Cryptococcus* or Mucorales [289]. There are several different available assays, but only Fungitell® (Associates of Cape Cod, Inc., East Falmouth, MA) is FDA approved for use in serum; no assays are FDA approved for testing of BAL samples. A threshold of ≥80 pg/mL is considered positive on the Fungitell® assay (product label), although other thresholds have been reported to have better diagnostic accuracy [324]. False-positive BDG results can occur in patients receiving albumin, intravenous immunoglobulin (IVIG), and other blood products [325–327].

Unfortunately, there are limited data regarding the diagnostic performance of serum BDG in children. With a high NPV, serum BDG is most valuable in excluding IFD in high-risk patients, including neonates, rather than identifying patients with true fungal infections [309, 328, 329]. A recent meta-analysis identified three studies in pediatric cancer or HSCT patients [309]. Among 226 children, 38 were diagnosed with proven/probable IFD and the diagnostic performance of BDG across these studies was as follows: sensitivity 50–83%, specificity 29–82%, PPV 17–49%, and NPV 84–96% [309].

BDG testing from BAL specimens is a potentially appealing approach to the diagnosis of pulmonary IFD. However, since airway colonization with fungi is common, results of studies have been poor. A meta-analysis of six adult studies that included 838 patients, 138 of whom had proven or probable IFD, found that BDG from BAL specimens had marginal diagnostic value [330]: pooled sensitivity of 52% and specificity of 58%. Salerno et al. reported that BAL BDG was inferior to serum BDG for the diagnosis of PJP in a cohort of 119 patients with HIV [331]. Based on available data, performance of BDG from BAL specimens does not appear to add value to serum BDG testing.

Parasitology

Introduction

Parasitic infections are extremely common in children around the world, especially in warm, low-income countries where sanitation is poor and housing is crowded [332]. Parasites can be classified as either protozoa (unicellular organisms) or helminths (multicellular worms), which are further categorized as nematodes (roundworms), cestodes (tapeworms), or trematodes (flukes). Most parasitic infections are acquired through the fecal–oral route, but several are vector borne, such as *Plasmodium* spp. (malaria) and *Trypanosoma* spp. (Chagas disease, African sleeping sickness) [333]. The frequency with which parasitic infections manifest pulmonary symptoms, and would be amenable to diagnosis via bronchoscopy, is highly variable across the myriad of organisms that cause human infections.

Many protozoan infections have pulmonary manifestations as the result of the disseminated forms of the disease: *Plasmodium* spp. [334], *Toxoplasma gondii* [335–338], *Leishmania* spp. [339], *Entamoeba histolytica* [340, 341], *Babesia* spp. [342], and *Trypanosoma* spp. [343]. In most cases, the pulmonary signs/symptoms are the indirect result of tissue damage (pneumonitis, pulmonary edema, pulmonary effusion,

and acute respiratory distress syndrome) rather than primary pulmonary disease [343]. However, some protozoa have been associated with bronchopulmonary infections directly. *Toxoplasma gondii* and other protozoa, such as *Balantidium coli*, *Cryptosporidium* spp., and *Microsporidium* species, have been reported as causes of pneumonia in immune-compromised individuals [336–338, 344–348]. *Entamoeba histolytica* has a predilection to form extra-intestinal abscesses, which can involve the lung [340], most often from extension of an amebic liver abscess [341]. Extremely rare cases of bronchopulmonary infections have also been reported with *Lophomonas blattarum* [349–351], a protozoa uncommonly associated with human disease. In very rare instances, ingestion and/or aspiration of free-living amoeba (*Acanthamoeba* spp., *Balamuthia mandrillaris*) can result in invasive respiratory tract infection [352].

All helminths have life cycles that include an egg, larval (one or more), and adult stages [332]. Entry into the human body occurs in one of three ways: ingestion of eggs or larvae, direct inoculation of skin either by larvae, or through an arthropod vector [332]. When direct or arthropod inoculation occurs, larvae enter the systemic circulation eliciting eosinophilic inflammation in various tissues, including the lung [343, 353, 354]. Similarly, after acquisition via ingestion, larvae can penetrate intestinal mucosa and enter the bloodstream, or they can migrate directly to the lung or pleura [343, 353, 354]. An example of the helminthic life cycle is shown for *Necator americanus* in Fig. 9.5 [355]. As the larvae migrate through the lung, eosinophilic pneumonia or pneumonitis may develop, a condition called Loeffler syndrome [356]. Accompanying symptoms include cough, wheezing, and fever, and peripheral eosinophilia is common [356].

Several helminthic infections are associated with pulmonary manifestations (Table 9.3). *Schistosoma mansoni* [357, 358], *Ancylostoma duodenale* [354], *Necator americanus* [354], *Dirofilaria immitis* [359, 360], *Toxocara* species [361, 362], *Paragonimus* spp. [363], *Ascaris lumbricoides* [364], and the agents that cause filariasis (*Wuchereria bancrofti, Brugia malayi*) [353, 365] can induce diffuse, eosinophilic inflammatory responses that manifest as pulmonary symptoms (wheezing, cough). *Echinococcus* species form cysts, which are most often asymptomatic when present in the lung. However, cysts can manifest pulmonary symptoms as a result of airway compression or due to hypersensitivity reactions when they rupture [366–368]. *Ascaris lumbricoides* [369] rarely causes direct pulmonary infections following aspiration, although ascension of adult intestinal *Ascaris* roundworms from the esophagus can lead to tracheal obstruction and respiratory distress [370, 371]. *Strongyloides stercoralis* infections are generally limited to the intestinal lumen in immune competent individuals, but may cause a mild, transient respiratory illness secondary to Loeffler syndrome [356]. In immune-compromised patients, particularly those receiving steroids, a life-threatening hyperinfection syndrome can develop in individuals with *Strongyloides* intestinal infection/colonization in which the organisms penetrate the intestinal lining and migrate to numerous tissues including the lungs [372]. In this setting, larvae and adult parasites may be detected on BAL fluid [373, 374]. *Trichinella spiralis*, acquired from ingestion of undercooked pork, can form diaphragm and accessory muscle abscesses/infection leading to respiratory effort weakness and pulmonary symptoms, but does not directly cause lung infection [375].

While most parasitic infections are confined to the gastrointestinal lumen, many species invade the bloodstream as part of their life cycle [332, 333]. These infections can induce eosinophilic inflammation either systemically or locally after migrating to the lungs [354]. Protozoa can also cause disseminated infections that are associated with pulmonary manifestations, such as acute respiratory distress syndrome and pulmonary edema [343]. As a result, pulmonary symptoms accompany parasitic infections at varying frequency. Because few parasites cause solely pulmonary disease, bronchoscopy is a relatively limited tool in the diagnosis of parasitic infections. Diagnosis of parasitic infections is most often made via direct examination of stool or blood, or via serology [332]. The Center for Disease Control

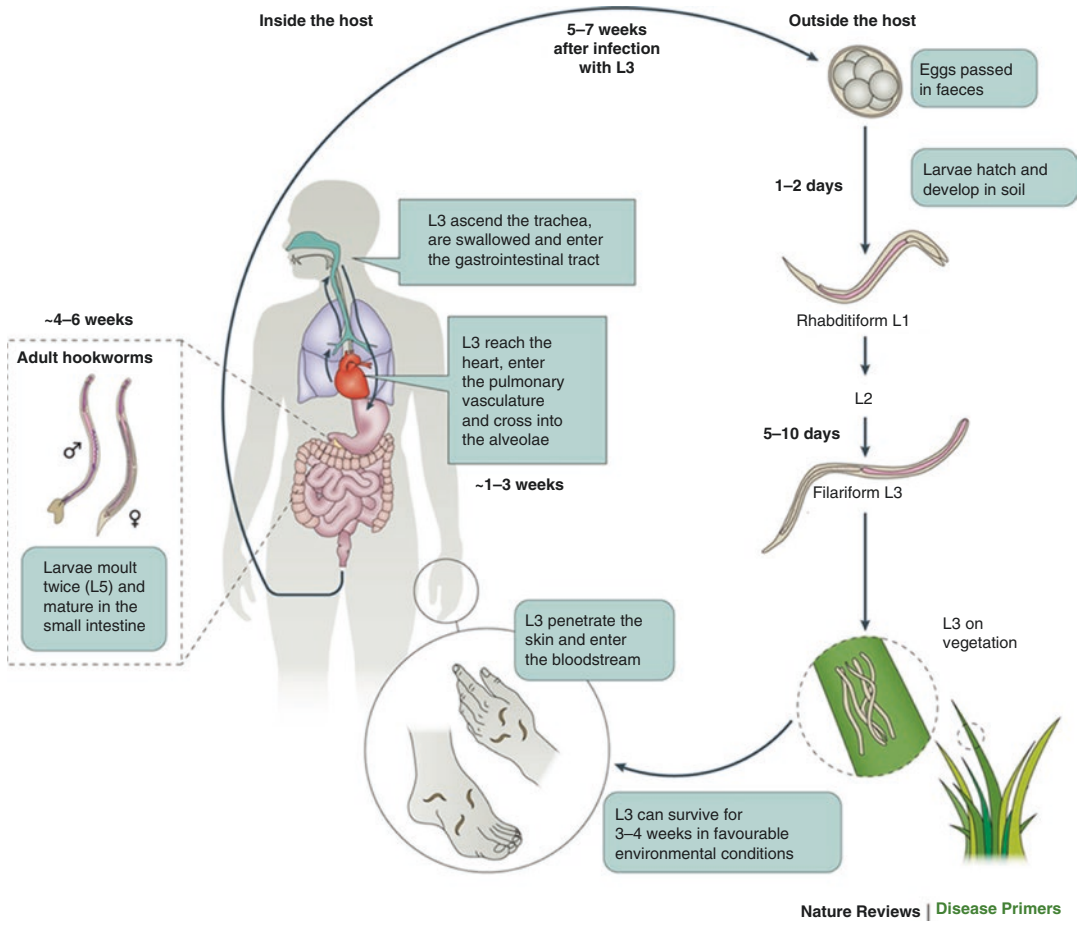

Fig. 9.5 Life cycle of *Necator americanus.* Hookworm eggs hatch in soil and rhabditiform (early) larvae molt twice (first-stage larvae (L1) and L2) before becoming infective (L3). L3 accumulate in soil or on grass awaiting exposure to human skin (often the hands, feet, or buttocks), which they can penetrate. L3 then make their way to the peripheral vasculature, where they are passively swept within the bloodstream, first to the right side of the heart and then to the pulmonary vasculature. In the lungs, L3 exit from the alveolar capillaries into the bronchial tree, which they ascend to reach the pharynx, from which they enter the gastrointestinal tract to finally complete their migration to the small bowel. Once in the duodenum, immature L5 hookworms use "teeth" (*Ancylostoma* spp.) or cutting plates (*Necator* spp.) that line their buccal capsule to lacerate the mucosa and anchor themselves in position to facilitate feeding and avoid being ejected by gut peristalsis. As they begin to feed on blood, juvenile worms mature into sexually dioecious adult parasites. Mature adult male and female hookworms mate, and female hookworms produce as many as 10,000 eggs per day. Eggs are evacuated from the host via the fecal stream. The process from L3 invasion to patency (egg production) takes approximately 6–8 weeks for *Necator americanus* and possibly a similar period of time for *Ancylostoma duodenale.* (Reproduced with permission from Loukas et al. [355]. © Springer Nature 2016)

and Prevention (CDC)'s Division of Parasitic Diseases and Malaria (DPDM) serves as a national reference laboratory for diagnosis of parasitic infection in the United States. A list of diagnostic procedures and specimen handling requirements can be found on their website (https://www.cdc.gov/dpdx/diagnosticprocedures/index.html).

For patients with severe, persistent, or atypical respiratory symptoms, or in immune-compromised individuals, bronchoscopy may be useful. Demonstration of peripheral or pulmonary eosinophilia, or elevated IgE, in the right epidemiological context, can be suggestive of parasitic infection. The following sections highlight the utility of vari-

Table 9.3 Helminth infections with pulmonary manifestations

Helminth	Mechanism of pulmonary involvement	Notable features of disease	Diagnosis
Ascaris lumbricoides	Hematogenous spread; inhalation/ aspiration	Associated with pulmonary eosinophilia and Loeffler syndrome Rarely, adult worms in the intestine can ascend into the oropharynx and cause airway obstruction	Detection of eggs in stool by light microscopy is primary means of diagnosis Serology available Adult worms may be directly visualized if coughed up or causing airway obstruction
Dirofilaria immitis	Hematogenous spread	Cause of canine heartworms Transmitted by mosquitoes *D. immitis* is a causative agent of human pulmonary dirofilariasis Symptomatic infection in humans is very rare Usually presents as an asymptomatic, solitary, well-circumscribed, peripheral nodule without calcification ("coin lesion") on imaging	Histology of biopsied tissue Serologic and molecular testing (PCR) available
Echinococcus species	Hematogenous or lymphatic spread; rarely via inhalation	Also known as hydatid disease Cystic echinococcus is the most common form of disease Symptoms develop when pulmonary cysts compress bronchi or rupture causing a hypersensitivity reaction Cysts are most often unilateral and solitary	Chest imaging (x-ray, CT scan, MRI) in conjunction with epidemiologic exposure Serologic tests available Bronchoscopy limited since rupture of cysts can induce hypersensitivity response; biopsy of cyst membrane may aid in diagnosis
Filariasis [*Brugia malayi, Wuchereria bancrofti*]	Hematogenous or lymphatic spread; immune-mediated inflammatory response	Adult worms in the lymphatics can release microfilariae into the systemic circulation, which can get trapped in the pulmonary circulation/lungs Associated with pulmonary and peripheral eosinophilia; may be referred to as tropical pulmonary eosinophilia May present as cough (often nocturnal) and dyspnea Peripheral signs of filariasis (lymphedema) may be present	Serologic tests used most often Filarial antigen may be present in blood Microfilariae can be visualized in blood or urine
Hookworms (*Necator americanus, Ancylostoma duodenale*)	Hematogenous spread	Enter body via percutaneous penetration from soil Organisms exit the vasculature via the alveolar capillaries and ascend into the GI tract Associated with pulmonary and peripheral eosinophilia during migratory phase Moderate or heavy intestinal infection associated with iron-deficiency anemia and hypoalbuminemia	Microscopic or molecular-based tests of stool Bronchoscopy could be used to detect larvae migrating from bronchi to trachea, but rarely performed

(continued)

Table 9.3 (continued)

Helminth	Mechanism of pulmonary involvement	Notable features of disease	Diagnosis
Lophomonas blattarum	Direct tissue infection	Rare protozoa carried by cockroaches and termites Vast majority of cases reported from China	Identification of flagellated organism from respiratory secretions (bronchoalveolar lavage fluid)
Paragonimus species	Direct migration from GI tract or liver to pleural cavity	Human infection from consumption of under- or uncooked shellfish (crabs or crayfish) May cause eosinophilic, chylous, or cholesterol pleural effusions Pleural (chest pain, dyspnea) and pulmonary symptoms (cough, hemoptysis) are most common clinical manifestation of infection Peripheral eosinophilia very common	Serology most commonly used Direct visualization of eggs in sputum (most often), but also BAL fluid, pleural fluid, biopsied tissue PCR available
Schistosoma species	Hematogenous spread; immune-mediated inflammatory response	Acquired via percutaneous penetration Pulmonary symptoms the result of a systemic hypersensitivity reaction during migration (first weeks after acquisition) Symptomatic infection more common in nonimmune individuals (i.e., travelers) Chronic pulmonary disease can result from egg deposition incidentally carried via venous system to pulmonary tissues; granulomas formation around eggs can lead to pulmonary hypertension	Demonstration of eggs in stool or urine by microscopy is primary means of diagnosis; lower sensitivity in acute infection since testing may be performed before eggs are deposited Serologic tests available; more useful in acute infection among individuals at low risk for past infection (i.e., travelers); not useful in endemic locations Bronchoscopy has limited role due to low organism burden within airways
Strongyloides stercoralis	Intestinal penetration and migration to lungs	Infection is mild in immune-competent individuals Disseminated infection (hyperinfection syndrome) can develop among immune-compromised patients, particularly those receiving steroids Associated with pulmonary eosinophilia	Stool microscopy is primary means of detection Serology available and useful in screening transplant candidates at high risk for hyperinfection syndrome Bronchoalveolar lavage may reveal larval forms in hyperinfection syndrome
Toxocara canis, Toxocara cati	Hematogenous spread	Larvae penetrate host tissues, including lung, and induce tissue-specific eosinophilia Peripheral eosinophilia common Pulmonary involvement associated with cough and wheeze	Serology is a preferred method of diagnosis Eosinophilia and elevated IgE common Peripheral nodular lung opacities may be present on imaging Pulmonary eosinophilia may be detected via bronchoscopy but direct detection of organism very rare

ous laboratory methods for diagnosing parasitic infections of the lung via bronchoscopy.

Culture

Very few parasites are diagnosed clinically using culture systems. For a number of reasons (low organism burden, varying life cycle, long replication half-life), it is difficult to grow parasites from human specimens in the laboratory. Thus, for the most part, parasite cultures are limited to research settings. While certain techniques have been developed to isolate organisms for antimicrobial susceptibility testing purposes [376], these are not used routinely in the clinical setting. As a result, parasite cultures are not generally performed on specimens obtained bronchoscopically.

PCR/Nucleic Acid Testing

Molecular diagnostic techniques are useful for the diagnosis of many parasitic infections and may be replacing conventional, microscopic approaches, especially for protozoa. Although sensitivity is variable, PCR tests are highly specific, often to the species level [377], and serve as good confirmatory tests [377–379]. PCR can also be performed on a variety of clinical specimens. Most often, parasitic infections are diagnosed via testing on blood/serum [378–381] and stool [382, 383]. In patients with suspected pulmonary infections, however, PCR can detect localized infection when performed on lung tissue or abscess fluid, such as for *Entamoeba histolytica* [383–385], *Echinococcus* spp. [386], and dirofilaria [387]. PCR also can detect the presence of various pathogens on BAL fluid, including schistosomiasis [388], *Toxoplasma gondii* [338, 389], and microsporidia [390]. However, due to the low organism burden associated with most parasitic infections involving the lung and lack of validation of many parasite PCRs from respiratory tract specimens, some experts debate PCR's utility over conventional staining techniques [391].

PCR from sputum holds potential to be a non-invasive method of detection of malaria, even though DNA in sputum may be several hundred-fold lower than that in blood [380]. In a study of 327 febrile individuals from India, 187 were diagnosed with malaria via microscopic evaluation [380]. The investigators found that a nested PCR assay of sputum identified more than 87% of microscopically confirmed cases [380].

Antigen-Based Testing

Direct fluorescent-antibody (DFA), enzyme immunoassays (EIA), and immunofluorescence assays (IFA) have been used for diagnosis of a limited number of protozoa including *Cryptosporidium* and *Giardia* species [382] and *Entamoeba histolytica* [383]. Commercially available assays are available, are more sensitive than direct microscopy, and have been incorporated into many clinical laboratories [383]. However, their use from BAL fluid or lung tissue is limited.

Pathology/Cytology

Light microscopy is the primary means of diagnosing the majority of parasitic infections [332, 364, 383]. Although sensitivity and specificity of microscopy is highly dependent on the technical skill of the microscopist, it is gold standard diagnostic method in parasitology. For parasitic gastrointestinal infections, direct wet-mount preparation is used for microscopic examination of stool ova and parasite (O & P) samples, while concentration and staining techniques increase the visualization of eggs, larvae, and cysts in samples [382, 383]. Direct visualization of eggs or adult worms from stool is specific, but sensitivity varies based on organism burden in intestinal lumen. Sampling of multiple stool specimens increases the likelihood of detection [364]. Kato-Katz thick smear is recommended by the World Health Organization for identification of soil-transmitted helminth infections such as *Ascaris lumbricoides*, *Trichuris trichiura,* and hookworms, as well as *Schistosoma* species [392]. Microscopic examination of Giemsa-stained blood films is the conventional approach

for diagnosis of bloodstream parasites such as in malaria and babesiosis. Indirect fluorescent antibody techniques are also available for a variety of parasitic species from tissue specimens.

Histopathology is particularly useful for diagnosing parasitic infections of the lung. Depending on the life cycle and migratory pattern of the parasite, eggs, larvae, or adult organisms may be detected in sputum samples, BAL fluid, or lung biopsy specimens [384]. Microscopic examination of sputum can be used to identify eggs of *Paragonimus westermani* [393] or larvae of *Strongyloides stercoralis* [394–396]; more rarely, larvae of *Ascaris lumbricoides*, hookworms, and *Entamoeba histolytica* can be detected in sputum. *Paragonimus* eggs can also be identified in pleural fluid on H&E stain, while adult flukes can be visualized within cystic cavities [384]. *Entamoeba histolytica* lung abscesses and empyemas may contain trophozoites that can be visualized on hematoxylin and eosin (H&E) or periodic acid Schiff (PAS) stains [384, 397]. *Schistosoma* spp. eggs and *Dirofilaria immitis* worms elicit granulomatous inflammation and necrosis, respectively, and can be directly visualized within biopsied lung tissue [384, 398]. Echinococcal cysts also have characteristic pathologic features that can be amenable to diagnosis via fiberoptic bronchoscopy [368, 384, 399, 400]. In rare cases of filariasis, microfilariae can be visualized on examination of bronchial lavage fluid [401, 402].

Airway Microbiome

In the past 15 years, molecular strategies have emerged for diagnosing bacterial and fungal infections that are less targeted. Whereas the molecular strategies discussed above target specific pathogens, new culture-independent techniques take an approach more akin to culture, in that they cast a wide net for many potential microorganisms, and then use modern DNA sequencing techniques to identify potential pathogens. Culture-independent techniques have so far mostly relied on the specific amplification of bacterial or fungal genes from BAL samples. In order to cast a wide net, the target genes need to be conserved across multiple species, such that they can be amplified with a common set of PCR primers, but they must be distinctive enough to allow identification (classification) to the species level. The most commonly used target for bacteria is the gene that encodes the small subunit of the ribosomal RNA (16S rRNA gene). For fungi, the corresponding small subunit, 18S, does not have enough discriminatory power, and the internal transcribed spacer (ITS) between the small and large subunit ribosomal RNA genes is often used.

Direct amplification and sequencing of the bacterial 16S rRNA genes from BAL fluid offers the potential for more sensitive detection of potential pathogens as compared to conventional bacterial cultures, but it also offers the possibility of collecting information on multiple microbial species and their relative abundances at once, opening up the prospect of understanding the ecology of infection and colonization [58, 403–406]. This new capability has led to two kinds of diagnostic goals: (1) finding the pathogen or pathogens without culture, and (2) illuminating properties of the full microbial community that may be biomarkers or even the root cause of disease. This second goal has taken microbiology out of the field of infectious diseases and placed it in allergy/immunology, rheumatology, and other pulmonary diseases.

Only a handful studies have used BAL to examine the pediatric microbiota in disease states such as protracted bacterial bronchitis [407], cystic fibrosis [58, 80, 408, 409], and patients with other chronic lung disease or immunocompromise [58, 406]. In general, these studies have demonstrated a diverse microbiota and, similar to studies done in adults [405], a high sensitivity for detecting bacteria in the lower respiratory tract [58]. Several studies [406, 410–412], but not all [58], have identified specific microbial community profiles associated with certain diseases or disease categories. However, the increased sensitivity of this technique also comes with a new diagnostic challenge. Samples often yield lists of more than 15 bacteria, and the most abundant bacteria are not always the pathogens recovered on standard culture from the same samples [58]. A detailed understanding of the relationship

between relative abundance, overall abundance, and microbial composition will be needed to make this technique clinically viable.

Another important question is whether or not invasive bronchoscopy with lavage is required to obtain the necessary information about the lower respiratory microbiota. Several studies have compared the microbiota of the upper and lower respiratory tract and reported varying levels of congruence and overlap in these microbial assemblages [67, 406, 413–420]. More work on paired upper and lower respiratory samples, in different age groups and types of disease, will be needed to resolve this issue.

Most respiratory microbiota (or microbiome) studies have been done targeting the 16S rRNA gene, and therefore, they can only detect bacteria and archaea. Several studies have used BAL other studies targeting fungi [421–430].

Fewer studies have analyzed BAL fluid for viruses [422, 431–434], but several studies aimed at viruses have been done on other respiratory samples (reviewed in [435]), some from pediatric populations [436–440]. Because viruses can have either RNA or DNA as their genetic material and do not have a single conserved gene among them, shot-gun metagenomic techniques, in which all nucleic acids in a sample are sequenced, remain the only option. Thus, studying the virome usually requires isolating viral sequences from a sea of human and microbial sequence data. However, studies that use a metagenomic approach also hold the promise of a complete picture of the respiratory microbiome. Such techniques are still costly and remain untested in the clinical setting.

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