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# Current and Emerging *Legionella* Diagnostics for Laboratory and Outbreak Investigations

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## SUMMARY

Legionnaires' disease (LD) is an often severe and potentially fatal form of bacterial pneumonia caused by an extensive list of *Legionella* species. These ubiquitous freshwater and soil inhabitants cause human respiratory disease when amplified in man-made water or cooling systems and their aerosols expose a susceptible population. Treatment of sporadic cases and rapid control of LD outbreaks benefit from swift diagnosis in concert with discriminatory bacterial typing for immediate epidemiological responses. Traditional culture and serology were instrumental in describing disease incidence early in its history; currently, diagnosis of LD relies almost solely on the urinary antigen test, which captures only the dominant species and serogroup, *Legionella pneumophila* serogroup 1 (Lp1). This has created a diagnostic "blind spot" for LD caused by non-Lp1 strains. This review focuses on historic, current, and emerging technologies that hold promise for increasing LD diagnostic efficiency and detection rates as part of a coherent testing regimen. The importance of cooperation between epidemiologists and laboratorians for a rapid outbreak response is also illustrated in field investigations conducted by the CDC with state and local authorities. Finally, challenges facing health care professionals, building managers, and the public health community in combating LD are highlighted, and potential solutions are discussed.

## INTRODUCTION

In the summer of 1976, the Centers for Disease Control and Prevention (CDC) in Atlanta, GA, responded to a sudden, explosive epidemic of febrile illness with pneumonia among attendees of the American Legion conference in Philadelphia, PA (1). With heightened public awareness due to "swine flu" earlier that year and mass vaccinations potentially on the way (2), front-page headlines dubbed this new threat "Legionnaires' disease" (LD) (3). A total of 32 people, with at least 20 epidemiologists, led by David Fraser, were mobilized from the CDC, the largest team sent to the field for any outbreak in the center's history to that date, to work with local and state agencies (4). The investigation uncovered 221 suspected cases of this unusual respiratory disease from conference attendees and bystanders in and around the convention hotel (including cases originally labeled "Broad Street pneumonia"); ultimately, 34 individuals died (5, 6). Amid widespread

**Citation** Mercante JW, Winchell JM. 2015. Current and emerging *Legionella* diagnostics for laboratory and outbreak investigations. Clin Microbiol Rev 28:80–118. doi:10.1128/CMR.00029-14.

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doi:10.1128/CMR.00029-14

speculation on the nature of this idiopathic disease, scientists ruled out toxicity from >30 heavy metals and infection by 77 known pathogens; however, attempts at growing the culprit organism on 14 different media and in 13 virologic hosts were initially unsuccessful (7). By December of that year, Joseph McDade and coworkers isolated what proved to be a new genus of bacteria from guinea pigs exposed to patient lung tissue, subsequently naming it *Legionella* for the American veterans' association (i.e., the American Legion) (7–9). Culturing and detection of *Legionella* were originally hampered by fastidious growth requirements and variable bacterial staining in infected tissues (2, 6, 10), but once the organism was isolated, scientists at the CDC developed tools and methods to reexamine historical collections and past outbreaks with similar presentations. Those scientists found clinically associated *Legionella* isolates from as far back as 1947 (11, 12) as well as patient seroconversion in two previously unsolved disease clusters: the first was in Washington, DC, in 1965, where 14 of 81 infected individuals died (7, 13), and the second was a nonpneumonic outbreak that occurred in Pontiac, MI, in 1968, where no deaths were reported among 144 cases (7, 14). The latter condition became the clinically and epidemiologically distinct "Pontiac fever," an acute, shorter-duration, self-limiting, flu-like illness with a high attack rate, which accounts for <1% of *Legionella* infections reported in the United States (6, 10, 15). The term "legionellosis" is commonly used to describe both the pneumonic and nonpneumonic forms of this disease. As we now know, these two syndromes may coexist within an exposed population (Fig. 1) (16–18), but it is unclear whether Pontiac fever is one potential outcome in the spectrum of disease severity or whether it is due to the presence of nonviable legionellae, amoebal pathogens, and/or high levels of bacterial endotoxin (19–23).

The 1976 Philadelphia outbreak spurred the swift development of serological methods for LD diagnosis and laboratory techniques for cultivating and isolating the bacterium. Today, many of these original diagnostic tests are still commonly used in laboratories; however, current and emerging proteomics- and nucleic acid-based methods afford significant improvements and expanded capabilities in this area. The goals of this review are to (i) briefly provide background for the physiology and ecology of legionellae, (ii) examine the historical and current state of *Legionella* detection and diagnosis in clinical and nonclinical laboratory settings and identify gaps and areas in need of improvement or modernization, (iii) highlight advances in molecular-based detection methodologies developed in the last decade that are being applied and implemented in clinical and research settings, (iv) describe recent and past *Legionella* outbreaks to capture their complexities and diversity while emphasizing the importance of cooperation between epidemiologists and laboratorians during these intensive investigations, and (v) discuss current challenges facing health care professionals and administrators, facility managers, public health officials, and laboratorians in addressing rising LD rates in the coming decades.

## PHYSIOLOGY AND ECOLOGY

Legionellae are aerobic, Gram-negative, non-spore-forming gammaproteobacteria. *Legionella pneumophila*, the most widely studied species, undergoes a phenotypically distinct biphasic life cycle that alternates between a nonmotile, replicative phase and a virulent, flagellated, transmissive phase (23–25). The bacterium displays dramatic pleomorphism, demonstrating coccoid, bacillary (~0.3- to 0.6-μm by ~3-μm), and/or long filamentous (~8- to 50-μm) forms that are influenced by temperature, available nutrients or metabolites, growth environment (e.g., inside amoebae), and medium type (7, 23, 26–29). *Legionella* spp. are ubiquitous in freshwater habitats, including rivers, lakes, streams, ponds, hot springs, and subsurface waters, and are naturally part of microbial ecosystems (Fig. 1) (30–33). Several species have also been recovered from composts and potting mixes in the United States, Australia, and the United Kingdom and in the soil of Thai farmland (34–41). At present, there are ~56 distinct *Legionella* species (and many unnamed species) encompassing at least 70 serogroups, approximately half of which have been isolated from, or detected in, clinical specimens, but all species are regarded as potential human pathogens (42, 43).

In the environment, legionellae can be associated with complex biofilm communities, where the bacterium likely transitions to a motile, planktonic stage; all legionellae studied have the ability to infect and replicate inside freshwater amoebae, which commonly consume biofilms (44–49). The fastidious *in vitro* nutritional requirements of *L. pneumophila* (27, 50) originally contradicted findings of its recovery from low-nutrient, highly competitive, polymicrobial environments (23, 31, 51, 52). However, it soon became clear that the unique physiology of legionellae was primarily adapted for survival and replication within numerous protozoan genera, including *Acanthamoeba*, *Naegleria*, *Hartmannella*, and *Tetrahymena* (24, 53, 54), and secondarily as a free-living or biofilm-associated aquatic bacterium. Their association with amoebae, in the vegetative or cyst form, may induce virulent bacterial phenotypes, assist in distribution, and provide protection from harsh or bactericidal environmental conditions, such as excessive heat and chlorine (55–65).

Legionellae thrive in tepid water (25°C to 37°C) but may propagate at temperatures above and below this range and may even survive at growth-restrictive temperatures of <20°C and >55°C (66, 67). A recent controlled, pilot-scale hot water distribution study confirms what many hospital, hotel, and cruise ship operators have reported: legionellae may persist and quickly recolonize potable water networks even after multiple rounds of heat shock (70°C for 30 min) and biocide treatment (68). An extensive collection of case studies and research articles dating back >30 years demonstrates the adaptability and potential of legionellae to colonize man-made aquatic environments (Fig. 1), from the initial point of water treatment (69–73) to private homes (74–78), hospitals (79–83), restaurants (84, 85), bath houses (86–89), hotels (90–92), and, eventually, wastewater facilities (93, 94). These studies underscore the resiliency and persistence of legionellae.

## EPIDEMIOLOGY AND DISEASE

Legionnaires' disease is a respiratory illness caused by inhalation of *Legionella*-containing aerosols generated by showers, faucets, air-conditioning cooling towers, whirlpool spas, and fountains, among others (Fig. 1). Legionellae are frequently isolated from natural waters, but these sources are typically not implicated in direct transmission, with one possible exception being natural hot springs adapted for human bathing, such as public baths, which are popular in Japan and Taiwan, among other locations (86, 95–101). Aspiration of water containing *Legionella* has also been suggested to be a common transmission route (102), although the frequency with which this occurs is unclear. Reports have suggested that immunocompromised patients in health care settings

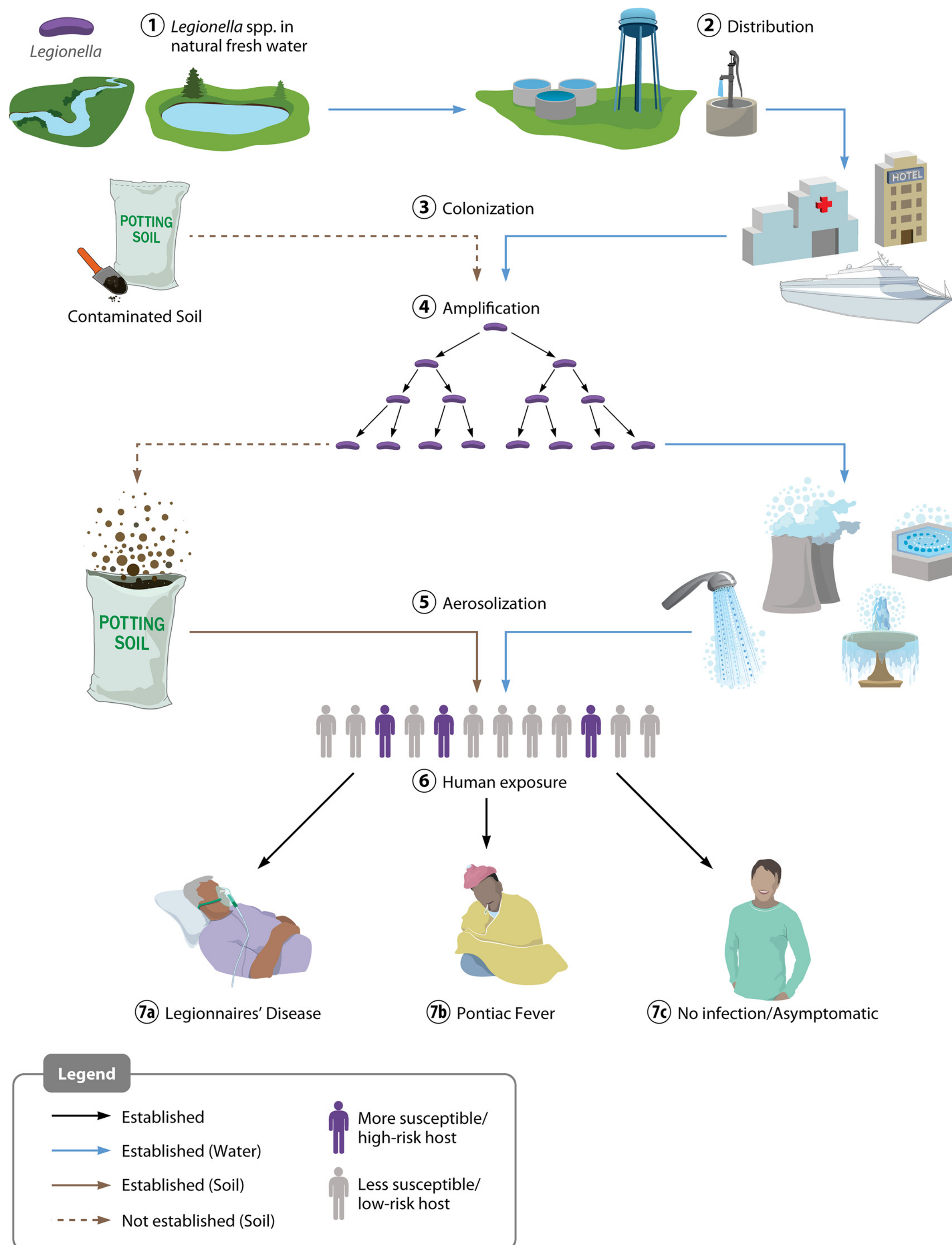


**FIG 1** Route of *Legionella* dissemination from natural waters to development of Legionnaires' disease and/or Pontiac fever. *Legionella* from freshwater sources (1) is distributed at low concentrations from points of water purification (2) to colonize downstream local plumbing networks and cooling systems (among other sites) (3) and amplifies under permissive environmental conditions (4). Subsequent aerosolization (5) exposes a human population, which may include individuals with increased susceptibility (6), leading to a potential disease spectrum. More susceptible individuals (due to age or underlying medical conditions) are at a higher risk of LD than those less susceptible, and both groups are at risk for Pontiac fever. The route of LD caused by contaminated soil is less well understood but also appears to involve aerosol exposure.

may be at risk from contaminated respiratory equipment (103–108); in these specific instances, the use of sterile potable water may be advised (109–111).

Humans are considered incidental (and dead-end) hosts, whereby legionellae infect and replicate within alveolar macrophages. The resulting illness may manifest as a febrile disease characterized by pneumonia and possible bacteremia (112–115). Together, *Legionella* spp., *Mycoplasma pneumoniae*, and

*Chlamydophila pneumoniae* represent the "atypical" branch (i.e., not *Streptococcus*) of nonzoonotic bacterial respiratory pathogens, responsible for ~22% of cases of community-acquired pneumonias (CAP) in the United States and Canada and up to 28% of cases worldwide (116). Legionellae alone are responsible for at least 8,000 to 18,000 hospitalizations every year in the United States, accounting for 2 to 9% of all pneumonias, a statistic also reflected in international studies (e.g., CAPNETZ), and yet, it may be an underestimation (116–119). The majority of LD cases occur in the summer and fall and more commonly affect males >50 years of age who have lung disease or immunosuppression (Fig. 1) (15, 82, 120, 121). Additional risk factors for legionellosis include smoking; recent travel; and underlying medical conditions such as diabetes, cancer, AIDS, end-stage renal disease (121, 122), and, potentially, human cellular Toll-like receptor 6 (TLR6) mutations (123). Notably, the reported LD incidence has increased substantially since 2000 for all age groups and U.S. geographic regions, with the median age for disease trending younger between 1990 and 2005 (15, 124–126). While this increase may in part reflect a true rise in the number of LD cases, a combination of factors may be contributing, including increased diagnostic testing, changes in case reporting methods, and expansion of the vulnerable elderly population. The influence of changes to national water quality standards or adjustments in medical insurance reimbursement patterns is likely minimal (15, 124). Interestingly, sporadic LD has been linked to higher-than-average atmospheric temperatures and increased rainfall (or humidity) in several studies, and while a mechanism is not clear, standing road water may play some part in infection (127–134). A similar increase in the overall European LD incidence has also been documented over the past 20 years but with a possible plateau being reached in the late 2000s (120, 135).

Clinical and radiographic presentations of LD are virtually indistinguishable from those of other, more common forms of pneumonia (136). Rapid, laboratory-based or point-of-care (POC) testing is crucial for accurate diagnosis and improved detection of LD outbreaks, and it allows for confirmation of LD-inclusive empirical treatment, changes in drug dosage or duration, or targeting of alternative antibiotics active against *Legionella* spp. (10, 137–139). In addition, despite the sensitivity of *L. pneumophila* to commonly available antibiotics, LD is associated with greater CAP severity and a higher case-fatality rate (up to ~30%) than CAP from other atypical pathogens (121, 140–145). The U.S. LD case-fatality rate has decreased steadily since the mid-1980s (125), from a high of ~34% to 8% on average in the 2000s (15). Although European LD case-fatality data were not compiled before the mid-1990s, current estimates place these rates on par with those for the United States during the previous decade (135, 146–149). *L. pneumophila* serogroup 1 (sg1 or Lp1) is responsible for 70 to 92% of laboratory-detected legionellosis cases in the United States and Europe and ~50% of the cases in Australia (120, 125, 150). The remaining species and *L. pneumophila* serogroups, such as *L. pneumophila* sg6, *L. longbeachae*, *L. micdadei*, and *L. bozemanii* [*sic*], account for most of the remaining disease, with the exception that *L. longbeachae* is the source of ~30 to 55% of LD cases in Australia and New Zealand; however, proportions vary greatly depending on the state or territory (119, 125, 150–152).

Generally, the high percentage of Lp1 clinical cases is not reflected in the local environmental distribution of *Legionella*. Indeed, seroprevalence studies in many countries have demonstrated potential ongoing exposure to diverse *Legionella* serogroups and species, which typically contrasts with the greater clinical prevalence of Lp1 (153–160). As such, substantial effort has been devoted to characterizing Lp1 virulence determinants, such as secreted effectors or surface factors. The Lp1 lipopolysaccharide (LPS) serves as the basis for traditional serogrouping, and it contains a virulence-associated epitope (recognized by monoclonal antibody 2 [MAb2] or MAb3/1 of the internationally used monoclonal panels) which is dependent on a functional *lag-1* gene for synthesis (161, 162). This LPS modification is strongly associated with Lp1 clinical disease and predominates in outbreak strains but is less frequently found in environmental Lp1 isolates (163–166; CDC, unpublished data). At present, it is not completely understood why this single serogroup and the *lag-1* genotype are responsible for the majority of clinical cases; potentially, some strains of *Legionella* may be especially pathogenic to humans, easily aerosolized, or more suited to colonization of anthropogenic water distribution systems (33, 163–170). One notable exception to the dominance of *lag-1*-expressing strains in LD cases is the Lp1 OLDA/Oxford (*lag-1*-negative) subgroup, which currently makes up 43% of all clinical isolates in Israel (171).

Unlike *Mycoplasma* and *Chlamydophila*, person-to-person *Legionella* transmission has never been reported, and community-acquired legionellosis is typically associated with man-made structures that generate water aerosols (Fig. 1), such as fountains (84, 85, 172–176), building water systems (75, 79, 177–184), cooling towers (185–193), and whirlpool spas (194–198), among others. U.S. surveillance data from 2001 to 2010 have consistently included *Legionella* as one of the leading causes of drinking water-associated outbreaks, accounting for 58% of cases in 2010 (199). While large-scale outbreaks, such as the 1976 Philadelphia epidemic or the recent and likely recurring Pittsburgh Veterans Health Administration Hospital incident (200, 201), attract national attention, the majority of LD cases are isolated and sporadic; from 2000 to 2009, outbreak-associated LD accounted for only 4% and 9.3% of all legionellosis cases reported in the United States and Europe, respectively (15, 114, 135, 146–149). Domestic and international travel-related disease is an increasingly recognized and significant category of infection; travel-associated legionellosis represented 19 to 24% of LD cases reported in the United States and Europe during the previous decade (15, 135, 146–149, 202, 203), with Lp1 predominating in both regions (204–213).

## DETECTION AND DIAGNOSIS

Procedures for the diagnosis and management of LD require important methodological distinctions from the identification of *Legionella* spp., serogroups, and sequence types (STs) for epidemiological investigations. While complementary, these related activities serve different objectives. Properly informed LD treatment does not necessitate discrimination beyond the genus level because all *Legionella* species tested are sensitive to commonly prescribed macrolides and fluoroquinolones (e.g., azithromycin and levofloxacin), which are active against and recommended for community- and hospital-acquired infections (214–220). Unlike CAP caused by *M. pneumoniae* or *Streptococcus pneumoniae*, acquired antibiotic resistance has never been reported for any *Legionella* strain, although a recent study reported a single clinical isolate displaying azithromycin and ciprofloxacin resistance outside the wild-type range (221–223). A follow-up study detailing the molecular basis for this resistance was not able to determine if mutations (in the *gyrA* gene) arose before or after the antibiotic

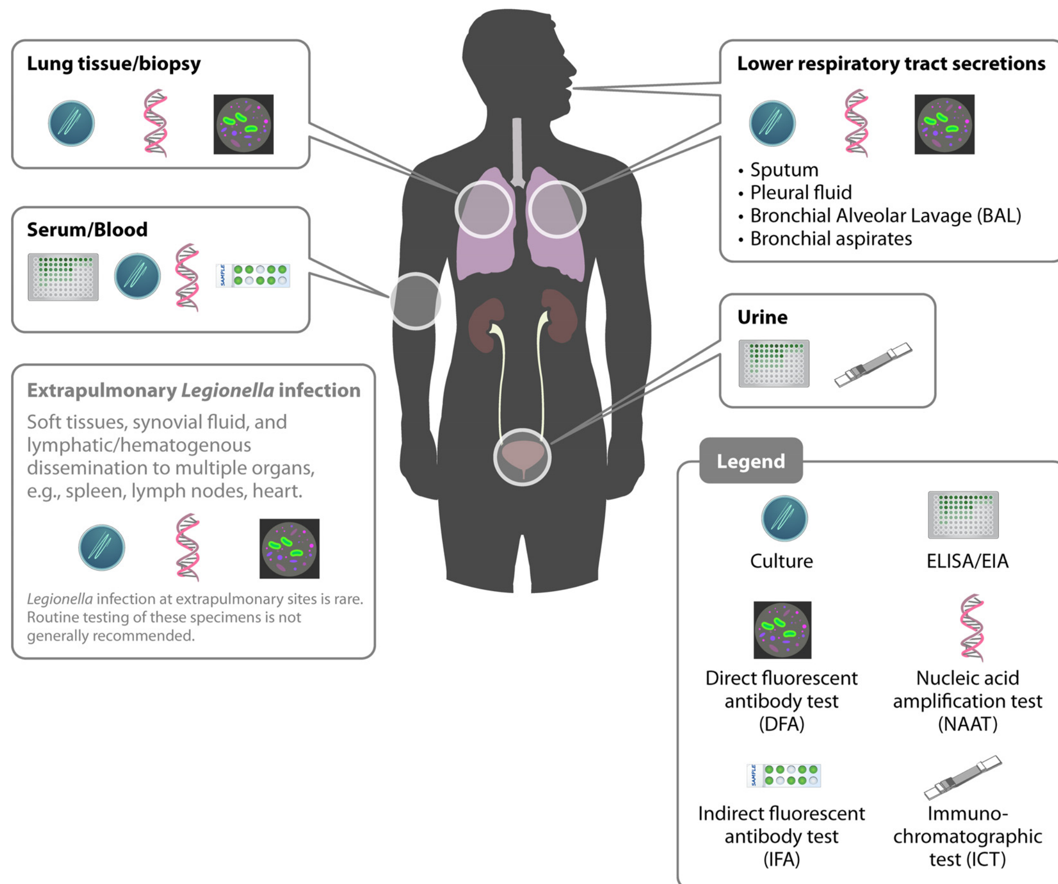


**FIG 2** Specimen types, diagnostic tests, and anatomical locations for determining a potential current or recent *Legionella* infection. Some assays are applicable to multiple specimen types, such as culture and nucleic acid amplification. In general, the success of detecting *Legionella* is dependent on the severity of disease, specimen integrity, technical proficiency of the laboratory, and particular test characteristics, as listed in Table 1. Additional recent emerging methods and technologies may also be used, such as mass spectrometry, but they may not be widely available or accessible. Note that *Legionella* infection at extrapulmonary sites, such as soft tissues or organs (e.g., spleen and heart), is rare.

was administered (224). Of particular interest, a next-generation macrolide (the first fluoroketolide), solithromycin, is currently in phase III clinical trials and, at least *in vitro*, appears to be highly active against Lp1 (225). In contrast, while timely processing is still important, epidemiological investigations that link one or more disease cases to common environmental exposures must employ more thorough approaches for identifying shared phylogenies between clinical and environmental strains. These techniques may include traditional antibody-based assays or more recently developed nucleic acid amplification tests (NAATs), such as PCR.

Early in the field's history, a limited set of culture- and antibody-based methods was used for both clinical and epidemiological investigations (e.g., direct fluorescent-antibody [DFA] assay); the later commercial development and widespread adoption of a *Legionella* urine antigen test (UAT) largely eliminated many of these assays from the clinical repertoire. Fortunately, nucleic acid molecular technologies introduced in the late 1980s and early 1990s, such as PCR and DNA sequencing, proved valuable for advancing both LD diagnostic and epidemiological capabilities (21, 226–229). Current LD case classification is based upon a combination of factors, including displaying clinically compatible symptoms (e.g., fever, myalgia, cough, and pneumonia), supporting epidemiological information, and positive laboratory findings (Fig. 2). In the United States and Europe, a positive laboratory result from a UAT, bacterial culture, and/or paired serology (i.e., indirect fluorescent-antibody [IFA] assay or enzyme-linked immunosorbent assay [ELISA]) for Lp1 defines a clinical case. Furthermore, detection of *Legionella* antigen or whole bacteria in respiratory secretions, tissues, or fluids by DFA, detection of seroconversion (4-fold or higher increase in titer) to non-sg1 serogroups, non-*pneumophila Legionella* species, or multiple species using pooled antigens, and/or detection of *Legionella* nucleic acid supports a suspected or probable case in the United States (230–233). Definitions for a probable case assignment in the European Union differ slightly from those in the United States, because European standards do not specify the minimum titer increase for seroconversion and they allow a single high antibody titer for assessing disease status.

Prospective and retrospective epidemiological studies may also use these case-defining laboratory techniques in addition to slide/serum/latex agglutination methods, monoclonal antibody (MAb) typing, and nucleic acid molecular methods such as *mip* gene sequencing, sequence-based typing (SBT) (234, 235), and/or PCR, both conventional and real time. Evidenced by its popularity in the peer-reviewed literature, *Legionella* nucleic acid detection is

being increasingly recognized, standardized (236), and implemented in the laboratory for rapid LD diagnostics and detection. The following sections detail the major categories of *Legionella* tests (Fig. 2) offered in clinical settings and at reference laboratories such as the CDC, emphasizing their purpose, benefits, and drawbacks as well as highlighting emerging technologies and procedures (Table 1). Information for clinicians and health departments, preferred diagnostic assays and collection procedures, and detailed protocols for most environmental techniques described here can be found on the CDC *Legionella* website, as listed in Table 3.

#### Microbiological Culture

Culture and isolation remain the "gold standard" for *Legionella* detection and LD diagnosis (51, 114). Acceptable culture specimens include those from the lower respiratory tract, such as sputum, pleural fluid, bronchial aspirates, and bronchial alveolar lavage (BAL) fluid (Fig. 2) (237). Lung tissue and biopsy specimens are also appropriate for attempting culture. Less conventional specimens include those from extrapulmonary sites, such as soft tissues, joint fluids, and blood (51, 113, 238). Reports of *Legionella* infection at these sites are rare, as is recovery of an isolate. In these instances, culture should be attempted only when other etiologies have been ruled out. Among all potential specimens for culture, sputum is generally most commonly sought, although a significant proportion of LD patients produce little or no sputum for culture analysis (51, 239). The sensitivity of detection of *Legionella* by culturing of clinical specimens is highly variable, ranging from <10% to 80%, and recovery is dependent on the sample type as well as the experience and technical proficiency of laboratory personnel (239). Legionellae grow on several types of complex artificial media; however, the most successful medium and procedure include buffered charcoal yeast extract (BCYE) agar containing 0.1% α-ketoglutarate with L-cysteine incubated at 35°C in a humidified, 2.5% $CO_2$ atmosphere (50, 240, 241). Most isolates demonstrate growth in 3 to 5 days, but non-*pneumophila Legionella* species and occasionally primary-specimen isolates may require considerably longer incubation times, sometimes up to 2 weeks (238, 242, 243). Despite extended growth periods, obtaining an isolate provides numerous advantages in allowing greater characterization and further epidemiological studies.

Not surprisingly, it is generally easier to isolate bacteria from patients with severe LD (due to increased bacterial burden) (119), and several methods can be employed to isolate *Legionella* from nonsterile specimens, such as sputum, or from heavily contaminated environmental sources, such as air-conditioning cooling towers (239, 244). Semiselective procedures enhance *Legionella* recovery in the presence of competing flora (from both clinical and environmental samples), including brief acid and heat exposure and/or the addition of glycine, polymyxin B, cycloheximide, and vancomycin to the growth media, to which legionellae are naturally resistant. Most legionellae are cysteine auxotrophs (the exceptions being *L. oakridgensis*, *L. jordanis*, and *L. nagasakiensis*, all of which may adapt to cysteine-deficient media after serial passage [245, 246]); thus, cysteine biplates can be used to quickly screen potential *Legionella*-like colonies (50). To date, at least 16 named and 6 undesignated species exhibit yellow-green, blue-white, or red-pink autofluorescence under long-wave (365-nm) UV light (238, 247–251; CDC, unpublished) when cultivated on BCYE or non-charcoal-containing medium. Most reported cases of LD are associated with *L. pneumophila*; however, currently, 8 of the 12 named species exhibiting blue-white and red fluorescence are also linked to human disease (81, 249, 252–256). Therefore, even uncommon environmental species should be considered potential human pathogens, especially for at-risk populations.

Clinical and hospital-based laboratories are a critical link in the chain of *Legionella* detection, diagnosis, and possible remediation. Ideally, attempting *Legionella* culture in all suspected cases for confirmation and further analyses should be the desired goal (214). Additionally, the concomitant development of alternative, culture-independent diagnostics should continue. Given its central role in LD investigations, the ongoing exchange of training and knowledge is encouraged, to sustain *Legionella* culture proficiency in the laboratory, especially since a sharp decline in the frequency of culture diagnosis has been reported in the United States, from >60% in the early 1990s to 5% on average from 2005 to 2009 (15, 125). The remarkable lack of laboratory expertise for *Legionella* isolation was confirmed by a College of American Pathologists survey, in which one-third of clinical laboratories were unable to grow a pure *Legionella* culture (138). The European Union has also experienced a large drop in overall culture-based detection, from 18% in 1996 to 12% in 2011 (120, 257). These statistical averages can be misleading since culture recovery can vary significantly among European Union member states. For instance, from 2007 to 2008, LD was diagnosed by culture in Spain and Italy in 0.45% and 1.7% of cases, respectively, while a higher culture confirmation rate of 15 to 40% was reported in Austria, France, Denmark, and the Netherlands (135, 203). A concerted effort by laboratorians and administrators is clearly needed to improve culture-based confirmation practices, preferably in conjunction with other tests based on nucleic acid amplification or UATs.

Until recently, there was no formal external laboratory accreditation program in the United States for environmental *Legionella* detection, making it difficult to assess the competency and proficiency of testing laboratories. To fill this gap, the CDC established the Environmental *Legionella* Isolation Techniques Evaluation (ELITE) program (see Table 3) (258). This free program, which began in late 2008, enables commercial, governmental, and hospital-based laboratories in the United States and abroad to evaluate their *Legionella* isolation techniques by using standardized, blind samples. Certification as an ELITE member requires biannual proficiency testing, whereby laboratories must successfully isolate and identify *Legionella* from heterogeneous aqueous mixtures. There are also similar programs in Europe for assessment of competency in the detection of *Legionella* in water samples (such as the "*Legionella* Scheme" administered by the Health Protection Agency of the United Kingdom). Given the complexity of patient specimens and existing regulatory oversight, there is no equivalent U.S. proficiency program specifically for clinical laboratory *Legionella* culture; however, third-party proficiency testing and certification for clinical bacteriology laboratories, required by the Clinical Laboratory Improvement Act (CLIA), may include *Legionella* as an unknown for culture and identification.

#### Urinary Antigen Test

The UAT has dramatically outpaced other laboratory methods for diagnosis, representing 82% and 97% of the diagnostic tools used for LD confirmation in Europe and the United States, respectively (15, 125, 144, 146, 257). The popularity and ubiquity of the UAT

are attributed to its speed, relatively low cost, uncomplicated procedure, ease of sample collection, commercial availability, and FDA clearance (CE marking in the European Union). *Legionella*-specific urinary antigens can be detected in the majority of *L. pneumophila* infections shortly after clinical symptoms appear (2 to 3 days) and may be excreted for several days to >10 months, even during antibiotic treatment and after disease resolution (244, 259, 260). For most cases, however, *Legionella* antigen is no longer detected in urine 1 to 2 months after therapy. The UAT was initially an in-house method for *Legionella* antigen detection (261–263) and was commercially developed as a radioimmunoassay in the late 1980s and early 1990s (264, 265).

Currently, the *Legionella* UAT is available from several vendors in two main formats: a 96-well plate-based enzyme immunoassay (EIA), or an ELISA, and a rapid, immunochromatographic test (ICT), in a card- or strip-based format, similar to a home pregnancy test (also known as a lateral-flow test). The most commonly used rapid ICT and EIA formats are highly specific for *L. pneumophila* (between 95 and 100%), with sensitivities from 70 to 90%, depending on whether urine is artificially concentrated; however, only five tests are FDA cleared for sale in the United States (Alere BinaxNOW *Legionella* urinary antigen card, Binax *Legionella* urinary antigen EIA, SAS *Legionella* test, Bartels *Legionella* urinary antigen ELISA, and Meridian Tru *Legionella*) and only for the detection of Lp1 (http://www.fda.gov/) (266–269). It is widely recognized that EIAs from some manufacturers exhibit cross-reactivity for various non-sg1 *L. pneumophila* serogroups and thus may detect a wider variety of pathogens. However, sensitivities among commercially available tests for non-Lp1 LD are highly variable and generally much lower than those for Lp1-associated disease when assayed with urine from patients with confirmed LD (267, 270–275). Importantly, the sensitivities of most similar-format UATs are generally equivalent, regardless of the manufacturer (267–269, 276–279), and they all allow for rapid assessment and patient treatment (280–282), unlike culture or serology, which may take days to weeks (139). Overall, the card-based ICT is a rapid, simple, qualitative assay for basic laboratory or POC use, while the EIA format is quantitative, may offer comparatively higher sensitivity and specificity, and is more suited for larger clinical, reference, or research laboratories (283).

Over the past 30 years, the development and implementation of UATs have greatly benefited LD patients by significantly improving diagnosis rates and thus allowing timely treatment (146, 257, 280). The LD-associated mortality rate decreased dramatically (~77%) in the United States from 1985 to 2009, along with a rapid increase in DFA and culture-based detection in the early 1980s, the mainstream introduction of the *Legionella* UAT (in the 1980s and 1990s), and updated guidelines (in the 1990s) by the American Thoracic Society and the Infectious Diseases Society of America for coverage of *Legionella* in empirical antibiotic therapy for CAP (15, 125, 284, 285). Several developments may have contributed to this changing diagnostic and treatment landscape, including standardized *Legionella* growth media, more readily available and sensitive DFA reagents, novel and rapid urine-based assays, and greater LD awareness by health care practitioners; additional data suggest that the superior sensitivity of the UAT over that of culture may have also allowed the detection of cases with milder disease and an inherently higher survival rate (125). In parallel with declining mortality, disease attributed to non-sg1 and non-*pneumophila Legionella* species decreased by 79% (125), suggesting that Lp1 is overrepresented in current estimates of LD. While the *Legionella* UAT is a valuable tool, sole reliance on this one diagnostic test may result in significant numbers of undetected LD cases (51, 125, 271). It is unclear whether this dramatic decrease in non-sg1-associated infections is in any way attributable to fewer actual cases. Surveillance conducted in the United States before and during the increase in UAT popularity suggests that the proportion of LD associated with Lp1 is variable year to year and ranges from 50 to 91% when only culture-confirmed cases are included (121, 125). More recent international surveillance and laboratory data suggest that a simple decrease in the non-Lp1 burden is not to blame. Denmark employs a more comprehensive diagnostic and testing regimen that relies on culture isolation and NAATs at levels well above the rest of the European Union and U.S. averages (120, 135). In the period from 1996 to 2006, approximately one-third of LD cases were culture confirmed in Denmark, which revealed Lp1 in only 60% of cases on average, similar to U.S. rates before the decline of culture techniques (271, 286). At present, it is uncertain if Denmark is burdened with higher-than-average enviromental levels of non-Lp1 (170); seroprevalence studies are suggestive of wide-ranging and diverse *Legionella* exposure in this country but fail to clarify questions surrounding environmental distribution (160, 287, 288). Regardless, enhanced surveillance and identification in Denmark likely detect a broader spectrum of LD caused by less common clinical serogroups and species, for which the current Lp1-specific UATs are not sensitive.

A closer analysis of Danish research also reveals an alarming trend: mortality rates for all non-sg1 LD patient groups were higher than those for any Lp1-infected population (i.e., MAb2 positive or negative) (271). Similarly high levels of mortality were observed in the United States between 1980 and 1989 for patients infected with *L. pneumophila* sg6 (121). Moreover, the reported survival rate is low (73%) for cases who are culture positive but UAT negative (125), a pattern which is more likely for non-Lp1 infections. Three plausible explanations for the apparently higher mortality rate in these subgroups include (i) preexisting patient immunosuppression leading to increased susceptibility to all legionellae and potentially higher inherent mortality, (ii) misdiagnosis and treatment delay due to the previously discussed UAT serogroup limitations (286), and/or (iii) increased non-sg1 *Legionella* pathogenicity. Given this historical overview of shifting diagnostic trends in the United States and recent international data suggesting a higher non-Lp1 clinical prevalence, it is reasonable to assume that significant underdiagnosis of non-Lp1 LD has occurred due to an overreliance on current-generation UATs.

Healthy populations across geographically diverse parts of the United States, Europe, the Middle East, Australia, Japan, South Korea, China, and former Soviet states display a large variation in seroprevalence for all legionellae, which additionally supports a reexamination of local *Legionella* exposures and the value of Lp1-specific UATs for diagnosing most LD cases (153–159, 289–303). This underscores a pressing need for the development of more inclusive *Legionella* rapid diagnostics; to be useful in a basic laboratory or POC setting, a pan-*Legionella* UAT would ostensibly be as simple to perform as current tests while detecting additional species and serogroups with similar efficiencies. Such a test would be invaluable, especially in regions with potentially higher environmental levels of non-sg1 *L. pneumophila* (e.g., the southwestern United States) and in countries where non-*pneumophila Le-*

**TABLE 1** Past, current, and emerging diagnostic tests for *Legionella* and LD[a]

| Test or diagnostic method | Specimen type(s) | Assay time to result (sample collection time) | Information provided by positive assay result | Use for confirmative or presumptive LD diagnosis (U.S.) | FDA-cleared or -approved *in vitro* diagnostic test or reagents commercially available | Advantage(s) | Disadvantage(s) | Sensitivity (%) | Specificity (%) | Notes |
|---|---|---|---|---|---|---|---|---|---|---|
| Culture and isolation | Sputum, respiratory secretions or tissue, and, more rarely, blood, synovial/joint fluid, or soft tissues | 3–14 days for growth plus cysteine biplate test | Together with testing for cysteine auxotrophy, identification of bacteria belonging to the *Legionella* genus | Confirmatory for *Legionella* species | Yes | Can detect all *Legionella* serogroups and species; supports epidemiological investigations | Long incubation and growth times; greater success in experienced laboratories; different specimen types associated with variable sensitivity | <10–80 | ~100 | |
| Urinary antigen test | | | | | | | | | | |
| EIA/ELISA | Urine | 3–4 h (negligible) | Infection by *L. pneumophila* sg1 only | Confirmatory for *L. pneumophila* sg1 only | Yes | Relatively rapid; availability of sample; may give positive results for long periods even after antibiotic treatment | Only FDA approved for Lp1; less sensitive for other serogroups; variable *Legionella* antigen excretion | 70–90 | 95–100 | |
| ICT | | 15–30 min (negligible) | | | | | | | | |
| Serology- and antibody-based assays | | | | | | | | | | |
| IFA (slide and ELISA formats) | Serum | 2 h–1 day (3–10 wk for paired sera) | Detects increase in antibody titer against several *L. pneumophila* serogroups | Confirmatory for *L. pneumophila* sg1 but presumptive for other serogroups | Yes | Useful when pathogen is not cultured; retrospective epidemiological studies; inexpensive | Does not provide timely, POC information; single titers can be misleading because of high preexisting seroprevalence; not specific for unknown strains or species; technically demanding, and results are subjective; not all cases seroconvert; potential cross-reactivity issues | 40–80 | 95–100 | Acute- and convalescent-phase sera collected 3–10 wk apart; sensitivity is lower early in disease |
| DFA | Sputum, respiratory secretions or tissue, blood | ~2 h (negligible) | *Legionella* serogroup and/or species discrimination | Presumptive | Yes | Inexpensive; commercially available reagents for identification and typing | Cross-reactivity may complicate interpretation; technically demanding | 25–75 | 95–100 | Can also be used for serogrouping and species identification |

| Col 1 | Col 2 | Col 3 | Col 4 | Col 5 | Col 6 | Col 7 | Col 8 | Col 9 | Col 10 | Col 11 |
|---|---|---|---|---|---|---|---|---|---|---|
| Slide agglutination | Culture isolate | 1–2 h (same as for culture) | *Legionella* serogroup and/or species discrimination | Not applicable | Unknown | Inexpensive; commercially available reagents for identification and typing | Cross-reactivity may complicate interpretation; requires a culture isolate | ≥89 | ≥97 | Other variants include latex and serum agglutination; test performance typically based on *L. pneumophila* detection only; likely less sensitive for non-*pneumophila Legionella* species |
| MAb blotting | Culture isolate | 1–2 h (same as for culture) | *Legionella pneumophila* sg1 and subtype discrimination | Not applicable | No | Simple procedure for *L. pneumophila* sg1 subtyping | Limited availability; requires a culture isolate | Unknown | Unknown | The EMD Duopath and Vircell VIRapid ICT devices report *Legionella* identification at specificities and sensitivities of ~93–100%; limited availability |
| Molecular assays | | | | | | | | | | |
| PCR (conventional and real time) | Any sample from which nucleic acid can be isolated | 4–6 h (negligible) | Identification and typing of *Legionella* species and *L. pneumophila* sg1 | Presumptive | No | Rapid; inexpensive; sensitive and specific; validated protocols widely available | Not FDA approved; can be sensitive to inhibitors; requires sophisticated and expensive equipment | 30–100 | 95–100 | Sensitivity and specificity dependent on assay design and specimen source |
| MALDI-TOF mass spectrometry | Culture isolate | ~15 min (3–14 days for initial growth) | Identification of bacteria as *Legionella* spp. | Not yet addressed | Yes | Rapid; inexpensive for established facility and trained personnel | Requires sophisticated and expensive equipment; serogrouping/subtyping not possible; requires a culture isolate | 90–99[c] | Unknown | Sensitivity dependent on species analyzed; pure-culture isolates are used |
| Isothermal amplification | Any sample from which nucleic acid can be isolated | ~1 h (negligible) | Identification of bacteria as *Legionella* species and *L. pneumophila* sg1 | Presumptive | No | Rapid; inexpensive; less sensitive to inhibitors than conventional PCR | Not FDA approved; still in early stages of development; not widely available | ~100[b] | >90[b] | Performance and efficiency dependent on assay design |

[a] ICT, immunochromatographic test; IFA, indirect fluorescent antibody; DFA, direct fluorescent antibody; EIA, enzyme immunoassay; MAb, monoclonal antibody, MALDI-TOF, matrix-assisted laser desorption ionization–time of flight; sg, serogroup.
[b] Very few studies for comparison.
[c] Compared to *mip* sequencing.

*gionella* species are documented in a significant proportion of LD cases (e.g., Thailand, Australia, and New Zealand) (271).

Regardless of the species or serogroup, clinicians should be particularly aware of *in vitro* diagnostic limitations when ruling out LD. A negative UAT does not necessarily exclude LD from consideration (304), because severe disease is more likely to yield a positive test (as opposed to mild LD), and results can vary greatly with time since exposure (141, 259, 305–307). These complications were confirmed in several research studies, where presumably low antigen excretion presented "delayed positive" ICT results (using the BinaxNOW *Legionella* ICT) observed at later time points (e.g., after 1 to 4 h of incubation) for samples initially giving borderline EIA absorbance measurements (267, 268, 276, 308). However, with some commercial products, this procedure may occasionally yield false-positive results, and manufacturers typically do not endorse this method of use (with some exceptions, e.g., Oxoid Xpect); in any case, results should be interpreted with caution if this method is performed (278, 309). In general, if initial UAT results are negative but the index of suspicion for LD remains high, clinicians are encouraged to perform testing multiple times over a longer period and/or to employ alternative testing modalities, such as PCR (283, 305), and additionally, concentrating urine can increase sensitivity without decreasing specificity (266, 279, 283, 306); however, boiling to reduce nonspecific interactions may be advised. These additional steps would likely abrogate the time advantages of the rapid ICT. Also, while results from a UAT may be sufficient for initial LD treatment, a culture from a patient specimen is still invaluable for epidemiological studies to mitigate further exposure from an environmental point source (214). From a public health standpoint, this cannot be overemphasized. Many outbreaks unfortunately result in a greater number of casualties due to the lack of an isolate from the initial patient(s), thus hindering an effective and timely public health response.

### Serological and Antibody-Based Assays

Serological testing for IgG and IgM antibodies against *Legionella* is a diagnostic tool that was critical in the original Philadelphia outbreak investigation (7, 9, 51) and one of the principal methods used for LD diagnosis in the early 1980s (125). While once popular, the number and scope of serological tests performed in the modern clinical laboratory have dropped significantly with the rise of standardized culture media and techniques and faster, more definitive analyses such as the rapid UAT and molecular methods (125). According to the European Centre for Disease Prevention and Control (ECDC) and the World Health Organization (WHO), the use of serology for LD confirmation in Europe declined from 61% to 6% on average in the period from 1995 to 2010, displaced by the faster, less technically demanding UAT (146, 257). Data from the U.S. passive surveillance system since 1980 and the Supplemental Legionnaires' Disease Surveillance System (SLDSS) between 2005 and 2009 highlight an equally dramatic 60% decrease in the use of serology, with <1% of case diagnoses currently relying on serology or DFA assays (15, 125). A similar trend was found in parts of Canada, where the probability of detection by serology or DFA has fallen precipitously with increased UAT usage (310). There are several obvious reasons for this change; even with the commercial availability of IFA assays and ELISAs for detecting patient seroconversion, serology is not a timely indicator of disease. Reliance on a 4-fold increase in antibody titer (to 1:128) between acute- and convalescent-phase serum samples taken 4 to 8 weeks apart means that the window for treatment has long passed (113). Furthermore, underlying medical conditions or immunosuppression may occasionally delay or prevent a 4-fold increase in titer from actually occurring, despite the existence of a bona fide infection (311–313). The majority (5.5% out of a total of 7%) of serology testing in the European Union is performed with a single, high convalescent-phase titer (120), which can be problematic, since prior exposure cannot be ruled out, even at titers of >1:256 (238, 314). A growing list of studies suggests that elevated Ig titers for *Legionella* can be detected in <1% to almost 30% of healthy individuals, depending on age, location, work environment, and, occasionally, gender (154, 155, 158, 288, 293, 297, 315). Of potential importance, the use of different in-house-developed and commercially developed IFA and EIA antigen preparations may complicate the interpretation of antibody titers for *Legionella*, especially across time and from different studies.

There are further challenges for serological assays: cross-reactivity may complicate the interpretation of results for non-*pneumophila* and non-sg1 *Legionella* infections (316–320), 20 to 30% of individuals with culture-confirmed LD never seroconvert based on the 4-fold rule (113), and proper interpretation of serological tests, such as IFA assays, requires extensive training and experience because results are often subjective and semiquantitative. As such, commercially available ELISA kits for detection of *Legionella* seroconversion may be increasing in popularity because they abrogate interpretational ambiguity through automation; however, the diagnostic accuracy of these tests is not yet established or agreed upon, and any trend toward their adoption has not been fully evaluated (216). Acknowledging these limitations, serology is still relevant for LD confirmation when the infectious agent cannot be isolated, and serology provides supportive data when corroborated by additional tests such as DFA or other immunohistochemical assays. Serology can also be valuable for retrospective epidemiological investigations, to identify patterns of disease, potential ongoing outbreaks, and general seroprevalence.

DFA assays, slide agglutination tests (SATs), and MAb screens are antibody based but not generally considered "serology" in the traditional sense because patient serum is not directly tested. SATs and MAb screens require a pure-culture isolate, while DFA assays can be performed on cultures, patient tissues, or secretions. Overall, their use in the clinical laboratory for *Legionella* respiratory antigen detection appears to be minimal, decreasing from a rate of 1% in 1996 to <1/10 of 1% in 2010 (146, 257). Interestingly, among ELITE member laboratories ($n = 141$), approximately half of clinical (43%) and commercial (54%) laboratories and most public health laboratories (65%) rely on MAbs or SATs for isolate or specimen confirmation, and a subset of laboratories use DFA assays for subtyping purposes (C. Lucas, personal communication). These numbers are not surprising given that ELITE-certified laboratories have a specific interest in *Legionella* environmental detection and typing.

DFA assays, SATs, and MAb blotting are useful for qualitative *Legionella* identification and typing at the species and serogroup levels. The tests benefit from being relatively rapid, inexpensive, and reliable, allowing strain comparisons across time with commercially available reagents (113), but similar to the IFA assay, they require a moderate-to-high level of laboratory expertise. Of particular note, MAb panels, such as the one developed by Joly et

al., that allow Lp1 subtype discrimination can be useful in research or clinical laboratories for epidemiological investigations (321–323). While this panel of MAbs is not widely available or sold commercially in the United States, a comparable collection, known as the Dresden panel, is distributed by a single research laboratory in Dresden, Germany (317). The CDC routinely employs all three methods alongside more recently developed nucleic acid-based amplification techniques for initial isolate screening and molecular typing for epidemiological studies. For instance, our laboratory now uses assays that rely on high-resolution melt (HRM) technology, along with alternative chemistries, to effectively identify clinically relevant *Legionella* species (CDC, unpublished data). We believe that these newer approaches support a more focused identification scheme than solely targeting conserved regions, such as 16S. One notable limitation to this, and all NAATs, is the inability to detect unknown or novel strains that may be present, and like the UAT, a negative DFA or IFA result does not necessarily exclude LD from the diagnosis and should not preclude attempted isolation by culture (113).

### Nucleic Acid-Based Molecular Diagnostics

Nucleic acid-based research for *Legionella* detection, diagnostics, and typing began in the mid-1980s (324, 325). Prior to the widespread adoption of PCR, scientists experimented with *Legionella*-specific DNA probes and commercial $^{125}$I-labeled *Legionella* DNA-RNA precipitations (Gen-Probe kit) (326, 327). The first report of PCR as a tool for *Legionella* detection came in 1989, when researchers from Stanford University combined PCR with Southern blot analysis to detect *Legionella* DNA spiked in water (227). Progress with PCR-based strategies continued into the 1990s for epidemiological studies with environmental samples. Both retrospective and prospective clinical diagnostic and epidemiological research validated this powerful new method in a variety of matrices, including water from cooling towers, rivers, and hot tubs as well as sputum, BAL fluid, serum, and urine (21, 229, 328–334). Real-time PCR gained popularity in the early 2000s, and although it requires technical expertise and complex, expensive thermal cyclers and software, many commercially marketed rapid environmental *Legionella* detection assays now employ this technology (Table 2).

The benefits of NAATs, including high sensitivity and specificity, rapid turnaround time, and widespread use, have validated this technology as a probable indication for clinical LD diagnosis (230–232). Isothermal amplification, conventional PCR, and real-time PCR (single and multiplex) protocols have been developed for *Legionella* detection and characterization, the latter enabling target quantification and bacterial enumeration. Since the mainstream introduction of real-time PCR, numerous groups have evaluated the efficacy of nucleic acid detection alongside culture and other established methods. Assuming proper bioinformatics and primer/probe design and stringency, most NAAT-based assays are highly specific (close to 100%), and the growing consensus is that the sensitivity of PCR (both conventional and real time) is equal to or greater than that of culture-based detection using specimens from the lower respiratory tract or environmental water samples (286, 335–344). Notably, the success of both PCR and culture for LD diagnosis is positively correlated with disease severity. However, culture demonstrates a greater decrease in sensitivity over the course of infection (due to antibiotic treatment and disease resolution) than PCR-based methods; thus, nucleic acid detection may be superior for diagnosing milder LD cases or detecting prior exposures (119, 336, 343). Additionally, PCR does not exhibit the apparent culture medium bias where BCYE or its selective variants favor the growth of particular *L. pneumophila* serogroups or *Legionella* species (345–348), and thus, NAATs may be more sensitive for the detection of all legionellae (339).

There is a growing list of commercially developed assays for *Legionella* nucleic acid detection (Table 2); however, only one has FDA clearance for clinical LD detection in the United States. A single test from Becton, Dickinson (BD Probetec ET *Legionella pneumophila*) that uses strand displacement amplification received FDA clearance in 2004 but is not currently available for sale in the United States (http://www.fda.gov). Commercial and in-house-developed NAATs are used in both clinical and environmental laboratories; however, the list of *Legionella* genes amplified is limited. The most common targets include a conserved segment of the rRNA genes for the 5S and 16S subunits, the 16S-23S spacer, and/or the macrophage inhibitor protein *mip*, found primarily in the genus *Legionella* and highly conserved in all *L. pneumophila* isolates (226, 332–334, 349–358). Several studies have also examined alternative chromosomal targets, such as *dotA*, *gyrB*, *dnaJ*, *wzm*, and *wzt* (340).

There is currently no consensus on the value of one gene or marker over another for *Legionella* NAAT development, with the exception that *mip* is typically used for *L. pneumophila* detection or general species identification. Ultimately, the selection of gene targets is influenced by the specific objectives of the testing laboratory, and thus, NAAT standardization may not be necessary or possible. One example in this respect is the CDC *Legionella* multiplex real-time PCR assay that was developed in response to the need for a prevalidated *Legionella* NAAT at U.S. state public health departments and partner laboratories in Thailand, Egypt, Kenya, and South Africa as part of the Global Disease Detection Program. The test was designed to simplify laboratory workflow for the simultaneous detection and typing of culture isolates, specimens, and contaminated environmental samples, with an internal control target (359, 360). This single-tube assay targets the *ssrA* (for all *Legionella* species), *mip* (for *L. pneumophila*), and *wzm* (for Lp1) genes.

In addition to detection and diagnosis, NAATs are commonly used for *Legionella* typing, mainly in conjunction with traditional MAb use or serology. Former and current nucleic acid typing methods include plasmid profiling, restriction fragment length polymorphism (RFLP) detection, pulsed-field gel electrophoresis (PFGE), ribotyping, arbitrarily primed PCR (AP-PCR) (or random amplified polymorphism DNA [RAPD] analysis), repetitive element PCR (rep-PCR), RFLP plus PCR (infrequent restriction site PCR [IRS-PCR]/amplified fragment length polymorphism [AFLP] analysis), and phylogenetic comparison of various *Legionella* species- and strain-specific genes, including *ftsZ* and *sidA*, among others (192, 228, 325, 361–370). *Legionella* species identification has relied largely on 16S rRNA gene or *mip* sequencing, while the common *L. pneumophila* sg1 Paris subtype can be further characterized by strain-specific, short, regularly spaced, palindromic sequences (spoligotyping) (333, 371, 372).

The current gold-standard *L. pneumophila* genotyping assay for epidemiological investigations is sequence-based typing (SBT), developed as a variant of multilocus sequence typing (234, 235, 373). SBT-based strain discrimination relies on the sequences of an ordered seven-gene collection (*flaA*, *pilE*, *asd*, *mip*, *mompS*,

**TABLE 2** Commercial nucleic acid amplification tests for *Legionella* DNA

| Company | Product name or description | Information provided by assay | Technology[a] | Sample source | Assay run time | Assay target(s) | Limit of detection listed | Availability |
|---|---|---|---|---|---|---|---|---|
| Qiagen | Mericon Quant *Legionella* species kit | Presence and quantification of *Legionella* species DNA | Real-time qPCR; probe based | Concentrated water | ~2 h | Not specified | 10 genomic equivalents/well | U.S. |
| Minerva Biolabs | Aquascreen *Legionella pneumophila* and *Legionella* species for real-time PCR | Presence and quantification of *Legionella* species and *L. pneumophila* DNA | Real-time qPCR; probe based | Concentrated water | ~2 h | 16S rRNA genes for *Legionella* species; *mip* for *L. pneumophila* | 10 genomic equivalents/well | International |
| ielab/Life Technologies | Kit for detection and quantification of *Legionella* | Presence and quantification of *Legionella* species and *L. pneumophila* DNA | Real-time qPCR; detection method unknown | Water | Not specified | Unknown | Not specified | Unknown |
| TIB MolBiol/ Roche | LightMix kit for *Legionella pneumophila* and *Legionella* species | Presence of *Legionella* species and *L. pneumophila* DNA | Real-time PCR; *Legionella* detection probe based, species detection method unknown | Not specified | 1–2 h | 16S rRNA genes for *Legionella* species; *mip* for *L. pneumophila* | 10 genomic equivalents/well | International |
| Diagenode Diagnostics | *Legionella* species and *Legionella pneumophila* real-time PCR | Presence of *Legionella* species or *L. pneumophila* DNA | Real-time PCR; probe based | Extracted human respiratory specimen | ~2 h | 16S rRNA genes for *Legionella* species; *mip* for *L. pneumophila* | 180 CFU/ml | International |
| Diateva | *Legionella* species quantitative kit | Presence and quantification of *Legionella* species DNA | Real-time qPCR; intercalating dye based | Not specified | ~2 h | Not specified | 5 cells/well | International |
| bioMérieux/ Argene | Chlamylege kit | Presence of *C. pneumoniae*, *M. pneumoniae*, and *Legionella* species DNA | Conventional PCR | Not specified | ~1 day | 5S-23S intergenic rRNA gene spacer | Not specified | Unknown |
| AES Chemunex/ bioMérieux | Adiacontrol *Legionella* | Unknown | Real-time qPCR; probe based | Water | <5 h | Not specified | 5 genomic equivalents/reaction | Unknown |
| Bio-Rad | iQ-Check Quanti *Legionella* species and *L. pneumophila* | Presence and quantification of *L. pneumophila* and *Legionella* species DNA | Real-time qPCR; probe based | Concentrated water | ~4 h | Not specified | Not specified | U.S. |
| Eiken Chemical Co., Ltd. | Loopamp *Legionella* detection kit E | Presence of *Legionella* species DNA | Loop-mediated isothermal amplification | Concentrated environmental samples | ~1.5 h | 16S rRNA genes | 60 CFU/test | U.S. and international |
| Genekam Biotechnology AG | *Legionella* PCR kits | Presence of *Legionella* species and *L. pneumophila* DNA | Conventional and real-time PCR; detection method unknown | Various | Not specified | rRNA genes | 2 CFU/reaction | U.S. and international |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Pall Corporation | GeneDisk Rapid Microbiology system | Presence and quantification of *L. pneumophila* and *Legionella* species DNA | Real-time qPCR GeneDisk system | Water | ~3 h | Not specified | 5 genomic equivalents/well | U.S. and international |
| Vircell Microbiologists | Speed-oligo *Legionella pneumophila* | Presence of *L. pneumophila* DNA | Conventional PCR combined with oligochromatographic test | Clinical samples | ~2 h | *mip* | Not specified | Unknown |
| Genycell | Duplica real-time *Legionella pneumophila* and *Legionella* species detection kit | Presence of *Legionella* species or *L. pneumophila* DNA | Real-time qPCR; probe based | Not specified | Not specified | 16S rRNA genes for *Legionella* species; 23S rRNA genes for *L. pneumophila* | Not specified | International |
| Life Technologies | TrueScience RespiFinder pathogen and viral identification panels | Presence of up to 19 different respiratory pathogens, including *L. pneumophila* | Multiplex ligation-dependent probe amplification and capillary electrophoresis | Multiple clinical sample types | Not specified | *mip* | Not specified | International |
| Genesig | PrimerDesign Genesig kit for *Legionella pneumophila* | Presence and quantification of *L. pneumophila* DNA | Real-time qPCR; probe based | Not specified | ~2–3 h | 16S rRNA genes | <100 target copies/reaction | U.S. and international |

[a] qPCR, quantitative PCR.

*proA*, and *neuA*), with the option of including a *neuA* homologue (*neuAh*) when the standard SBT primers fail to amplify the target in non-sg1 strains. SBT has the advantage of direct sequence comparison, which eliminates the interpretational subjectivity of non-sequence-based methods such as PFGE, which are prone to banding ambiguities over time or between laboratories (374). In support of SBT, the European Society for Clinical Microbiology Study Group on *Legionella* Infections (ESGLI) (formerly the European Working Group on *Legionella* Infections [EWGLI]) maintains an allele database (currently version 3.0) that allows querying of large sets of raw sequence data, delivering both an allelic profile and a final combined sequence type (ST) for each isolate (Table 3). The database is dynamic and continually updated with the addition of new allele sequences and STs.

SBT is traditionally performed on DNA extracted from culture isolates; however, several studies have demonstrated some success when standard or nested SBT was performed directly on nucleic acids extracted from patient tissues or fluids (375–378). This adds a much-needed tool to the *Legionella* typing repertoire. However, caution is stressed because the efficiency of SBT on culture-independent preparations varies widely with sample origin (e.g., sputum versus BAL fluid) and quality and also is typically much lower than that of pure isolate extractions. Laboratory expertise and the use of high-quality media can maximize bacterial growth from otherwise low-quality samples, yet if an isolate is not obtained because of prior antibiotic therapy or suboptimal shipping and storage, culture-independent SBT offers a potentially viable alternative.

*Legionella* nucleic acid-based detection offers significant advantages over serology and culture in terms of sensitivity and speed. However, there are several notable disadvantages and limitations. PCR may not be ideal for testing non-lower respiratory tract samples (e.g., urine and serum); at best, PCR sensitivity in these specimens only approaches that of the *L. pneumophila* UAT (286, 336, 343, 379–383). One inherent complication with all nucleic acid amplification methods is the difficulty in assessing bacterial viability. These methods do not discriminate between free nucleic acids, either in solution or amoeba associated; nucleic acids from dead or dying bacteria; and/or viable but nonculturable (VBNC) legionellae. This is evidenced in environmental studies that detected a higher level of bacterial DNA than was corroborated by culture and where the persistence of *Legionella* DNA after multiple rounds of remediation resulted in no detectable culture growth (183, 338, 341, 342, 384). As a consequence, PCR has a low positive predictive value (PPV) for legionellae compared to that of culture methods in environmental settings; conversely, PCR has a high negative predictive value for viable *Legionella* in the same samples (≥97%) (341, 343). Interestingly, PCR in clinical applications may have a higher PPV (than for environmental samples) despite the lower sensitivity with nonrespiratory specimens (379). Two potential remedies for the low PPV with samples from environmental sources include reverse transcription-PCR, amplifying labile RNA targets present in metabolically active bacteria, and the use of a cell-impermeant chemical, such as ethidium monoazide (EMA) or propidium monoazide (PMA), to inhibit PCR amplification from nonviable cells or extracellular nucleic acids (385–390). Notably, neither alternative protocol alone will discriminate VBNC legionellae; however, this cell population still poses a potential human health risk (391–394).

The expansion and development of nucleic acid amplification

**TABLE 3** Selected online *Legionella* resources[a]

| Organization(s) and/or institution(s) | Resource or reference | Topic(s) covered | Website URL |
|---|---|---|---|
| CDC *Legionella* Web pages | Main page has information for the public, clinicians, the media, health departments, and building and environmental professionals | Multiple topics, including disease facts, clinical indications, diagnostic tests, investigation tools, sampling protocols, position statements, and the ELITE program | http://www.cdc.gov/legionella/index.html |
| | ELITE program Web page | Details of the ELITE certification process for laboratories isolating *Legionella* from environmental water | http://www.cdc.gov/legionella/elite.html |
| | Epidemiological investigation tools | Diagnostic tests, case verification, and patient interviews; environmental assessment and sampling; decontamination (including hot tubs and cruise ships); and CDC assistance | http://www.cdc.gov/legionella/health-depts/inv-tools.html |
| | Environmental specimen collection and management | Procedures and protocols for environmental sampling and processing | http://www.cdc.gov/legionella/specimen-collect-mgmt/index.html |
| CDC and HICPAC | Guidelines for preventing health-care-associated pneumonia, 2003 (111) | Prevention of health care-associated legionellosis and other pneumonias | http://www.cdc.gov/mmwr/preview/mmwrhtml/rr5303a1.htm |
| | Guidelines for environmental infection control in health-care facilities (560) | Infection control in health care facilities, with some focus on *Legionella* | http://www.cdc.gov/mmwr/Preview/mmwrhtml/rr5210a1.htm |
| | Guideline for disinfection and sterilization in health care facilities, 2008 (587) | General health care disinfection and sterilization procedures, with notes on *Legionella* and amoebae | http://www.cdc.gov/hicpac/Disinfection_Sterilization/acknowledg.html |
| EWGLI/ESGLI Sequence Based Typing Database for *Legionella pneumophila* | Main page has links to multiple SBT resources | Multiple topics, including methods and protocols, SBT locus data, strain submission, sequence quality tools, and various query functions | http://www.hpa-bioinformatics.org.uk/legionella/legionella_sbt/php/sbt_homepage.php |
| EPA | Legionella: human health criteria document (588) | Multiple topics, including general information on *Legionella*, occurrence, human health effects, risk assessment, and environmental analysis | http://water.epa.gov/action/advisories/drinking/upload/2009_02_03_criteria_humanhealth_microbial_legionella.pdf |
| WHO | *Legionella* and prevention of legionellosis (589) | Multiple topics, including water safety plans, in-building distribution, and exterior cooling system assessment | http://www.who.int/water_sanitation_health/emerging/legionella.pdf |
| | Guidelines for drinking water quality, 4th ed. (590) | Drinking water risk assessment, with some attention toward *Legionella* | http://whqlibdoc.who.int/publications/2011/9789241548151_eng.pdf |
| | Water safety in buildings (591) | Water safety plans, risk management, and supporting information | http://whqlibdoc.who.int/publications/2011/9789241548106_eng.pdf?ua=1 |
| | Guidelines for safe recreational water environments, vol 2 (592) | Risk assessment and health hazards associated with water environments, including legionellosis | http://www.who.int/water_sanitation_health/bathing/bathing2/en/index.html |
| ASHRAE | Guideline 12-2000 (595)[b] | Guidelines for minimizing *Legionella* in building water systems | |
| | Proposed new standard 188 (593)[c] | Standard practices and operating procedures for building operators to prevent legionellosis associated with building water systems | https://osr.ashrae.org/Public%20Review%20Draft%20Standards%20Lib/Std-188P-PPR2%20Final%206%2010%202011.pdf |

| | | | |
|---|---|---|---|
| ECDC | ECDC legionellosis health topic website | Disease facts, news, epidemiological data, surveillance reports, recent publications, external resources, and *Legionella*-related events | http://ecdc.europa.eu/en/healthtopics/legionnaires_disease/Pages/index.aspx |
| | ELDSNet | ELDSNet information, case definitions, surveillance activities, operating procedures, forms, investigation guidelines, and an outbreak toolbox | http://ecdc.europa.eu/en/activities/surveillance/ELDSNet/Pages/index.aspx |
| Health and Safety Executive, United Kingdom | *Legionella* and Legionnaires' disease Web page | Multiple topics, including disease and treatment information, workplace risk exposure, employer responsibilities, recent news, and further *Legionella*-related resources | http://www.hse.gov.uk/legionnaires/index.htm |
| | Legionnaires' disease: the control of *Legionella* bacteria in water systems, L8, 4th ed. (594) | Approved code of practice and guidance for *Legionella* risk assessment, prevention, management, control, and monitoring processes | http://www.hse.gov.uk/pubns/priced/l8.pdf |
| Public Health England (currently merging with the Health Protection Agency) | Legionnaires' disease Web page | Multiple topics, including general disease and organism information, sampling and investigation guidelines, surveillance and epidemiological data, external proficiency testing scheme for *Legionella* (from a linked site), case definitions, and recent publications and reports | http://www.hpa.org.uk/Topics/InfectiousDiseases/InfectionsAZ/LegionnairesDisease/ |

[a] SBT, sequence-based typing; ELITE, Environmental Legionella Isolation Techniques Evaluation; EWGLI, European Working Group on Legionella Infections; ESGLI, European Society for Clinical Microbiology Study Group.
[b] Currently undergoing an update.
[c] Proposed standard not yet finalized.

methods offer important real and potential benefits to the field of *Legionella* detection and diagnosis. In comparison, serological or antibody-dependent assays create cross-reactivity and stability issues and require expensive investments in manpower, animal care, and time. Nucleic acid amplification technologies still necessitate specially trained personnel and sophisticated machines but are increasingly accessible to a wider array of laboratories on a moderate budget. The commercial availability of environmental *Legionella* nucleic acid detection kits (Table 2) and the abundance of research and methodology (244) mean that laboratories need not design, optimize, and implement a complex, "home-grown" strategy for testing. Additionally, there are some problems that nucleic acid molecular methods are more apt to solve; PCR is the only approach currently suitable for diagnosis of LD due to non-sg1 and non-*pneumophila Legionella* species in a time frame that could positively influence patient management (51).

Collectively, nucleic acid-based methods are valuable additions to LD diagnostic and detection schemes; however, the limitations inherent to NAATs (395), as discussed above, support the concurrent use of multiple testing modalities to increase the probability of successful detection. Combined with traditional confirmatory techniques, NAATs can augment diagnostic sensitivity for LD in clinical and epidemiological settings (119, 344), especially for less severe disease with lower bacterial loads, and can help define the full extent of disease burden (119).

## Emerging Methods and Technologies

Advancements in *Legionella in vitro* diagnostics are often derived from the application of novel approaches to existing assays or through *de novo* development of innovative technologies. Representing both methodologies are several established (mass spectrometry and real-time PCR-based TaqMan array cards) and emerging (immunomagnetic separation [IMS], isothermal nucleic acid amplification, high-resolution melt analysis, and whole-genome sequencing [WGS]) techniques that may enhance *Legionella* detection and characterization in various clinical settings. This will ostensibly improve outcomes during outbreak responses and epidemiological investigations.

Isothermal nucleic acid amplification is a general classification for DNA or RNA amplification at a constant temperature with minimal or no cycling, as is required for PCR (for reviews, see references 396 and 397). The major advantage of most isothermal techniques is rapid target detection (within 15 to 60 min) without the need for expensive, complex, and energy-demanding thermal cyclers. Among the various methodologies, nucleic acid sequence-based amplification (NASBA) and loop-mediated isothermal amplification (LAMP) have been used to detect *Legionella* DNA in clinical and environmental samples (130, 398–402). Numerous studies have employed isothermal amplification for detection and identification of viral (403–405), bacterial (406–408), and parasitic (409–411) pathogens, and various commercial kits and components are available. In particular, one company (Eiken Chemical Co., Japan) offers a *Legionella*-specific LAMP kit that is gaining popularity in environmental research and monitoring (130, 401, 402).

Microfluidic TaqMan low-density microarray cards (TAC), developed in the mid-2000s by Life Technologies, can quickly interrogate gene expression in various disease states (412–416). Researchers at the CDC adapted TACs for the simultaneous detection of >20 respiratory pathogens, including *Legionella* (417,

418); this custom array card is now the principal tool for identifying unknown respiratory disease outbreaks and is being piloted for population-based surveillance programs at several U.S. and international sites to define respiratory disease etiology and burden (419). TACs offer increased real-time PCR throughput (384 individual reactions) in a rapid, reproducible, and simple setup containing prespotted primer-and-probe combinations. This format has since been customized for larger field evaluations and for the detection of nonrespiratory syndromes (420–424).

Mass spectrometry (MS) is a mature yet still evolving technology adapted to the rapid identification and classification of clinically relevant pathogens (for a review, see reference 425). MS for *Legionella* identification was first performed in the late 1970s in combination with gas chromatography (426). The development of matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) MS has proven reliable for *Legionella* species identification and typing, although intraspecies serogroup discrimination is not yet possible (425, 427–430). Given a core facility with established expertise and equipment, MALDI-TOF MS is a fast (~10-min), inexpensive method for isolate identification that has recently received FDA clearance. Reference spectral databases are critical for MS chromatogram comparisons, and several have been created or improved to aid in *Legionella* typing (e.g., modified Bruker and Biotyper databases) (427, 429, 431).

IMS combines specific, whole-cell antibody recognition with magnetic bead-based purification for bacterial concentration. Published IMS research demonstrates sensitivity for legionellae in environmental and clinical samples, with or without filter concentration or DFA, at or above standard culture or fluorescence detection levels (351, 432–438). Further IMS development may be useful for rapid field, laboratory, or POC detection, although the specificity limitations of antibody-based isolation must be addressed (see "Serological and Antibody-Based Assays," above, for a discussion of limitations).

HRM curve analysis was originally proposed (439) and then developed to detect single-nucleotide polymorphisms (SNPs) within PCR amplicons (440–443). This technique may employ double-stranded intercalating dyes (e.g., SYBR-GreenER, EvaGreen, or SYTO9) or fluorescently labeled primers (Lux chemistry) in both initial real-time PCR and subsequent HRM analyses; alternatively, fluorescence resonance energy transfer (FRET)-based probes can be added after PCR for the secondary melt analysis. One variant of this technique employs a solid-state surface plasmon resonance sensor combined with gold-labeled probes (444). HRM analysis has been used in numerous studies since the mid-2000s on a wide range of human pathogens, including those within the genera *Campylobacter*, *Brucella*, *Leishmania*, *Bordetella*, *Clostridium*, *Mycobacterium*, *Mycoplasma*, *Chlamydophila*, *Cryptosporidium*, and *Staphylococcus*, among others (445–452). Recent studies have shown HRM analysis to be a powerful technique for characterizing antibiotic resistance and typing respiratory disease agents, including *M. pneumoniae*, *C. pneumoniae*, and *Chlamydophila psittaci* (453–459). To our knowledge, HRM analysis for intraspecies or interspecies *Legionella* discrimination has been included in only a few studies (460–463); however, this approach seems promising for future *Legionella* diagnostics or typing.

WGS is an increasingly popular and accessible technique with broad diagnostic potential for public health laboratories. The development of "next-generation" DNA sequencing platforms and reagents from a variety of manufacturers has dramatically decreased the time and cost of WGS over the past 10 years. A recent and growing pool of research demonstrates that these technologies are well suited for sequencing applications toward microbial identification and typing (464–470); examination of phylogenic relationships among pathogens for population-based, longitudinal, or retrospective epidemiological studies (471–488); identification of molecular bases for antibiotic resistance or virulence (489–501); discovery of DNA targets for diagnostic development (502, 503); and, less often, prospective surveillance or outbreak investigations (504–509). One of the largest hurdles to the adoption of WGS for a rapid response during public health emergencies is the development of an efficient bioinformatics pipeline for data analysis and interpretation, including comprehensive microbial reference libraries. Recently, WGS was employed in a retrospective United Kingdom pilot study and in real time during a *Legionella* outbreak investigation at an Australian hospital (510, 511). These initial studies confirm that WGS can provide high-quality typing and epidemiological data, although continued improvement in data analysis will undoubtedly be necessary to realize the maximum benefit of this approach. As part of the recently launched CDC Advanced Molecular Detection initiative (http://www.cdc.gov/amd), the CDC *Legionella* Laboratory Team is currently integrating next-generation sequencing technologies with enhanced bioinformatics capabilities for legionellosis detection and outbreak responses.

## CDC OUTBREAK INVESTIGATIONS: THE SYNERGY OF EPIDEMIOLOGY AND LABORATORY SCIENCE

### General Field and Laboratory Procedures for Outbreak Investigations

Between 1976 and 2013, CDC laboratories have assisted with the isolation, identification, and/or typing of legionellae in ~170 domestic and international outbreaks and thousands of sporadic cases (CDC, unpublished). In that same time frame, CDC personnel have participated in at least 98 *Legionella*-focused coordinated epidemic assistance investigations (Epi-Aids) (512, 513; CDC, unpublished). Our understanding of *Legionella* ecology, epidemiology, and disease is informed, in large part, by these investigations. Since 1976, Epi-Aid field work and subsequent research studies have been instrumental in defining the clinical description of LD and risk factors for contracting the disease as well as detailing the environmental growth, persistence, and epidemiological transmission of legionellae to susceptible populations (512). Concurrently, the CDC *Legionella* laboratory team and others have helped characterize bacterial physiology while developing diagnostics and procedures for disease and environmental detection. In order to detail and share how laboratory diagnostic and field detection methods are integrated with epidemiological investigations during high-profile *Legionella* outbreaks, the following paragraphs outline the general field and laboratory workflow. This includes sample collection and analysis as well as culturing and typing of legionellae to identify culprit strains and sources of environmental contamination. Procedures and protocols for assessment, collection, and testing can be found in Table 3, on the CDC *Legionella* website, as well as in a recent publication (514) and accompanying reports in the same volume.

The basic activities common to all outbreak-associated epidemiological field investigations assisted by the CDC *Legionella* lab-

oratory team include (i) environmental assessment and sample collection, (ii) culturing and testing of both clinical specimens and environmental samples, and (iii) phylogenetic and/or strain characterization of clinical and environmental *Legionella* isolates. During outbreak investigations, shared geography among disease cases typically defines the study area, while field personnel, led by epidemiologists and often including laboratorians, complete an extensive "on-the-ground" environmental assessment to identify potential sampling sites. The environmental assessment provides relevant information, such as the presence of water and air-handling systems that are capable of aerosol generation, but the specific number and type of samples collected are dependent on the size and complexity of the facility as well as the locations of reported LD cases. Investigations of localized LD clusters where patients share recent common exposures may require only 40 to 50 environmental samples from 30 to 40 sites, while outbreaks over larger, less defined areas in which potential exposure sources may not be obvious can necessitate >300 samples from 100 or more locations. As a standard practice, when possible, the CDC collects both 1-liter bulk water and biofilm swabs in sterile plastic containers from interior locations distal to incoming water, such as shower heads, faucets, hot water heaters, misters, decorative fountains, and spas, and from more central or proximal sites, such as incoming municipal or well water mains and hot recirculation supply lines. Air-conditioning cooling towers, which are historically associated with *Legionella* contamination (81, 245, 515–518) and LD outbreaks in the United States (187, 519–521) and internationally (186, 190, 521, 522), can support heavy bacterial growth, and all locations within the study zone are routinely sampled. At the time of collection, water is assessed for temperature, pH, and residual disinfectant (e.g., chlorine or bromine) before chemical neutralization with sodium thiosulfate. Samples are immediately packed and shipped in insulated containers to ensure minimal temperature fluctuation en route to the CDC *Legionella* Laboratory in Atlanta or an approved local testing facility.

In the laboratory, potable water is concentrated with 0.2-μm-pore-size polycarbonate filters, and nonpotable samples (e.g., from cooling towers or fountains) are both acid treated and directly plated onto BCYE solid medium with selective agents (e.g., antibiotics and/or glycine) (243, 514). Plates are incubated for up to 14 days, and primary colony isolates are replated and confirmed on cysteine biplates. If necessary, serogrouping is performed by MAb dot blotting, slide agglutination, and DFA analysis in some instances (238). *L. pneumophila* sg1 subgroups (e.g., Philadelphia and Benidorm, etc.) are assigned based on a previously developed MAb panel (321, 322). A representative subset of *L. pneumophila* isolates are typed by using the standardized ESGLI allelic sequence profiling method (234, 235, 321, 322). Non-*pneumophila Legionella* isolates and those of ambiguous type are identified by an in-house-developed real-time PCR assay followed by *mip* gene sequencing, if required (359, 371). Clinical specimens are interrogated by the same identification and typing procedures as those described above; an epidemiological linkage is established when SBT and MAb2 profiles match between patient and environmental isolates.

### High-Profile Legionnaires' Disease Outbreaks

U.S. federal, state, and local authorities as well as international organizations and foreign health ministries may formally request assistance from the CDC when LD is detected. A comprehensive Epi-Aid involves on-site support to health agencies in determining the scope of infection and the potential source(s) of disease transmission. These resource-intensive investigations are typically in response to an urgent public health threat and can garner local or national attention (e.g., a recent outbreak in Pittsburgh, PA, led to congressional hearings) (85, 92, 523). The CDC *Legionella* laboratory team is an essential part of the outbreak response and supports Epidemic Intelligence Service (EIS) officers and epidemiologists both in field investigations and with laboratory expertise. Several well-documented Epi-Aid investigations carried out by the CDC in the 1990s and 2000s are discussed below to illustrate the interdisciplinary nature of these events and to highlight the complexities and nuances in each type of outbreak investigation and, where applicable, emphasize important laboratory advances, such as new diagnostic or detection methods, that aided in the identification of a transmission source.

**Health care-associated outbreak in a long-term-care facility.** Older adults and persons with underlying medical conditions have an increased risk of *Legionella* infection; thus, hospital and nursing home settings are often implicated in outbreaks. In September 2004, the CDC responded to an LD outbreak in a long-term-care facility (LTCF) in Cherokee County, NC (193). In total, four residents of the LTCF and three local community cases were confirmed to have LD by the *Legionella* UAT; three of the seven cases were fatal. A comprehensive field investigation was undertaken at the LTCF, which centered on potential transmission from potable water; however, *Legionella* was not found in any sample collected from the facility. Focusing outside the LTCF, investigators conducted >250 interviews in the surrounding community in search of unidentified LD cases; three additional "confirmed" and two "possible" cases were detected. With the size and scope of the sampling area expanded, a local industrial cooling tower 0.4 km away from the LTCF was found to harbor multiple *Legionella* species and Lp1 monoclonal subgroups. In addition, *Legionella* DNA was detected (by PCR for the *mip* gene) on special filters fitted to rooftop air-handling units of the LTCF and was subsequently matched to the DNA of a cooling tower isolate. The proximity of the cooling tower to both the LTCF and the additional community cases was sufficient for a presumptive remediation of the site; unfortunately, patient respiratory specimens did not yield bacterial growth, which hindered the positive identification of a disease source. Notably, what was initially suspected to be institutional transmission (because two patients never left the LTCF) proved instead to be community based, requiring multiple rounds of interviews, sampling, and testing. The use of PCR for pathogen detection and typing provided laboratory flexibility in defining the likely transmission source and conduit (air-conditioning fresh-air intake). As highlighted in the published case description, illnesses at the LTCF represented sentinel events in a wider community exposure (193) and additionally underscored the complexities associated with *Legionella* outbreak investigations.

**Health care-associated outbreak in a hospital.** Immunocompromised individuals, especially transplant recipients on immunosuppressive regimens, are at an increased risk of developing LD due to Lp1 or other species and serogroups (524–528). In addition, case-fatality rates for LD are higher among older males and the immunosuppressed (121). In the first half of 1996, a cluster of health care-associated LD cases was identified at a Southwestern U.S. regional transplant center (529, 530). An initial in-house investigation and attempted remediation were unsuccessful in iden-

tifying and controlling the problem, and by summer, the CDC was requested to assist state and local officials. LD surveillance was intensified, and a retrospective study for previously unrecognized cases was initiated. A total of 25 culture-confirmed or potential health care-associated cases were found: 17 were identified with disease onset between 1987 and 1995, and 8 were discovered since then (from 1996 to the present). The calculated LD attack rate for all transplant patients at the hospital was 6%, and 12 patients ultimately died, for a case-fatality rate of 48%.

Urine samples were available for many patients; however, a commercially available UAT failed to detect *Legionella* urine antigens. Fortunately, a hospital policy promoting bacterial culture and typing combined with increased surveillance revealed infections by *L. pneumophila* serogroups 1, 4, 5, and 10 as well as by sg6, which represented more than half of the disease burden. An extensive field investigation of hospital potable water from case patient room showers and faucets as well as water softeners, hot water tanks, and private supply wells, among other locations, was conducted. Environmental samples processed at the CDC reflected the bacterial composition and diversity in prior clinical isolates; the CDC *Legionella* laboratory found the above-mentioned serogroups, with the addition of serogroup 11, *L. anisa*, and an undetermined species. *L. pneumophila* sg6 was detected in both the shower vapor of a case patient's room (by a specialized Andersen air-sampling device) and a carpet cleaner reservoir tank used in the bone marrow transplant unit. Hospital water supply systems can be extensive and complex, providing optimal conditions for *Legionella* growth and distribution. The difficulties and risks associated with water distribution for transplant medicine places added importance to disease monitoring and a rapid response in specialized hospitals or units. This particular outbreak highlights how field and laboratory techniques were critical in defining the sources of infection and linking them to clinical disease; a thorough epidemiological investigation helped identify common exposures, and by using PFGE, CDC laboratorians confirmed that the *L. pneumophila* sg6 strain found in the shower vapor and carpet cleaner was also responsible for most of the health care-associated LD cases.

**Commercially linked outbreak from an unlikely source.** Both potable and nonpotable water can support the amplification and transmission of legionellae to susceptible populations. During outbreak events, investigators face the daunting task of locating all aerosol-producing devices when relatively few common exposures are identified and disease is distributed over a seemingly wide area. Decorative water features are a frequent addition to public and private spaces and may serve as potential disease reservoirs, but they can be overlooked in water safety plans due to their unassuming size or lack of visible aerosol generation. In the fall of 2004, clinicians at Rapid City Hospital in South Dakota instituted increased diagnostic testing for seasonal CAP, which included a *Legionella* UAT, in order to more efficiently target antibiotic therapy. Eighteen cases of legionellosis were recognized in the summer and fall of 2005 due to this improved surveillance. The UAT was the primary method of diagnosis, while bacterial cultures were grown from respiratory specimens of 4 patients, and a 4-fold increase in serum titers was documented for one individual. Fourteen individuals were hospitalized, and one patient ultimately died. Beginning in June of the same year, the CDC began an extensive Epi-Aid response to this cluster of LD cases in Rapid City (85). After interviewing case patients and controls, ~300 targeted 1-liter bulk water samples and biofilm swabs were collected from 123 sites around the city and immediately shipped to the CDC in Atlanta and a commercial *Legionella* laboratory. The list of sites tested was extensive and citywide, including cooling towers, chillers, condensers, supermarket misters, showers, sinks, hot water tanks, municipal water systems and treatment plants, hot tubs, and multiple fountains. *Legionella* contamination was widespread, being identified in 35% of the locations tested.

*Legionella pneumophila* sg1 was the most common environmental isolate recovered from the study region (from 43 of 123 sources); however, a number of other serogroups and non-*pneumophila Legionella* species were also cultured, including *L. pneumophila* sg8, *L. anisa*, *L. bozemanii* [*sic*], *L. feeleii*, *L. rubrilucens*, and *L. spiritensis* (CDC, unpublished). It is not uncommon to find multiple potential transmission sources during outbreak investigations. Importantly, a promising epidemiological link among half of all case patients was recognized, and MAb subtyping and SBT analysis confirmed that the only environmental match to previous clinical isolates came from a small, unassuming, plastic decorative fountain in a local restaurant lobby; the outbreak promptly ended when the fountain was removed. Clusters of LD cases have been linked to decorative fountains both before and after this reported outbreak (84, 172–176); however, the current case was the first report of a small, low-aerosol-generating, decorative fountain as the source. Notably, the success of this investigation was dependent on epidemiological clues and clinical isolates available from four case patients, which enabled laboratory typing methods (MAb subtyping and SBT) to identify the exact transmission source among the large number of potential sites.

**Cruise ship-associated outbreak.** From 2005 to 2009, cruise-related LD cases accounted for ~5% of travel-associated legionelloses reported to the U.S. SLDSS (15) and 7.6% of cases on average in Europe during the same time frame (207–209, 211, 212). As previously reported (531), *Legionella* has become a problem for cruise ships in a similar fashion as for hotels; both must manage complex air-handling networks as well as potable and recreational water distribution systems with the potential for *Legionella* growth and transmission. Defective or improperly maintained onboard water systems may present an increased LD risk for the older average cruising demographic, which is between 55 and 61 years of age (532–534).

Identification of the largest cruise ship-associated LD outbreak to date (535, 536) began in mid-July 1994, when a New Jersey physician reported a cluster of legionellosis cases among three individuals returning from the same Caribbean cruise (537). Upon learning of three additional cases, CDC and New York State health officials distributed an epidemiological questionnaire to offloading passengers from the same vessel and provided a preliminary health warning to boarding travelers. After consulting with the ship's staff and cruise line representatives onboard, the CDC team began an environmental investigation by collecting 1-liter bulk water samples and biofilm swabs from sinks, showers, fountains, water heaters, storage tanks, and the whirlpool spa, among other sites. In addition, a number of tourist destinations were sampled at the ship's international port of call; all samples were shipped back to the CDC *Legionella* laboratory in Atlanta. A then-recently developed, commercially available *Legionella* PCR dot blot assay (EnviroAmp; Perkin-Elmer Cetus) detected *Legionella* species DNA at the majority of sites on the cruise ship (CDC,

unpublished), with *L. pneumophila* DNA in approximately half of the samples.

Among all sampled sites, the whirlpool spa and filtration system gave the strongest DNA hybridization signals, and a case-control study demonstrated a strong epidemiological disease association with the shipboard spa. Physical and chemical examination of the spa revealed large amounts of organic material in the sand filtration unit and no detectable bromine, which was required to prevent microbial growth. Importantly, *Legionella* culture growth was recovered from only this single location. State and international epidemiologists were immediately notified about the outbreak, and questionnaires were mailed to ~3,000 recent passengers in an effort to identify additional cases of disease. In total, 50 cases of LD, with a median age of 63 years, were identified from 9 separate cruises on the same ship during the spring and summer of 1994; one individual died. Sixteen cases were confirmed by UAT, serology, or both, and one *L. pneumophila* sg1 isolate was grown from patient sputum. The MAb subtype and AP-PCR patterns were an exact match between the single clinical isolate and the strain grown from the shipboard whirlpool spa.

This cruise-borne LD outbreak investigation is notable for the following two unique developments. (i) Only one location demonstrated bacterial growth despite positive *Legionella* DNA test results for the majority of environmental samples, including potable water. As detailed in "Nucleic Acid-Based Molecular Diagnostics," above, PCR is a powerful technique, but in most applications, it is not designed to discriminate live versus dead or VBNC bacteria. The exclusive use of this PCR-based method would have provided the false impression that most shipboard water was currently colonized, when, in fact, the whirlpool spa was the only detectable, and most likely, source of transmission at the time of sample collection, as confirmed by culture. (ii) It is a challenge to recognize cruise-related outbreaks due to the potentially long LD incubation period and the dispersal of passengers after a ship docks (531, 535, 538). Fortunately, during this event, a concerned physician prepared a careful medical and travel history for the first three identified LD cases (537), which allowed the recognition of wider disease transmission. The rapid reporting and response from local to state and federal levels mobilized resources to define the LD distribution and prevent further cases.

*Legionella* contamination onboard passenger and cargo vessels may be an unappreciated hazard, as detailed here and as documented in sporadic cases and outbreaks on smaller scales (531, 539–547). A recent survey of Norwegian naval vessels suggests that *Legionella* contamination is not isolated to passenger ships: researchers found *Legionella* species DNA in the onboard potable water of approximately one-half the naval vessels sampled, and they were able to culture *L. pneumophila* from a smaller subset of these ships (548). As described above, recreational baths and whirlpool spas can provide optimal conditions for bacterial growth and transmission and are among the nonpotable water sources most frequently associated with ship- and land-based outbreaks (18, 194–198, 531, 541, 549–556). To mitigate this waterborne hazard, the CDC offers public guidance for the safe operation and disinfection of recreational hot tubs, and the WHO provides literature for *Legionella* risk assessment in recreational environments (Table 3).

### Summary of Field and Laboratory Operations for Outbreak Investigations

Each LD outbreak presents a separate and distinct set of challenges for laboratory and field personnel. Some general parameters and guidelines are common to all outbreak responses (e.g., environmental assessment and sample collection), but no two situations require identical resources or activities. Even sampling in similar types of environments or scenarios requires an appreciation of individual water and air management systems, usage patterns, and, in some cases, weather and prevailing winds. The complex and sometimes unmapped water distribution systems found in both old- and new-building construction during a sustained outbreak at a regional transplant center necessitated sampling at hundreds of potential sites; unlike a typical transmission point source, the outbreak strain was systemic and was found in multiple areas. Investigations also quickly change with new information; the focus of sampling efforts can be influenced by an individual's recall during interviews, and outcome success is variable depending on the availability of clinical specimens. Several case patients in the South Dakota investigation remembered their potential restaurant exposure only after a reinterview or hearing about the outbreak in the media, and the implicated decorative fountain was not originally sampled because it was turned off during the environmental investigation and therefore was considered an unlikely source (85). The lack of a clinical isolate for epidemiological comparison in the North Carolina LTCF meant that transmission via the nearby cooling tower was presumptive as opposed to confirmed.

The LD outbreak examples mentioned above together illustrate several key points: (i) rapid, accurate transmission source identification during an outbreak response requires seamless cooperation between state, federal, and local health authorities and continuous communication among epidemiologists and laboratorians; (ii) molecular methods for *Legionella* detection and typing, such as PFGE, AP-PCR, real-time PCR, and SBT, increase diagnostic sensitivity and enhance strain discriminatory power over traditional techniques (e.g., SAT), but these results must be interpreted with care; (iii) a complete and thorough epidemiological investigation that identifies common points of exposure to LD cases is critical for narrowing down and defining potential transmission sources; and (iv) a patient culture isolate, or sufficient quantity of high-quality DNA from a specimen when no isolate is obtained, is necessary for linking clinical disease with an exposure source to enable focused remediation and to initiate public health efforts to prevent further infections.

## DISCUSSION AND FUTURE OF *LEGIONELLA* DIAGNOSTICS AND DETECTION

### Education, Awareness, and Reporting

From 2000 to 2011, the LD incidence rate more than tripled in the United States, increasing in every U.S. geographical region, sometimes dramatically (e.g., a >7-fold increase in the New England region) (15, 126, 557). A similar trend was documented in Europe, with a 158% increase over 9 years starting in 2004 (146, 148). This increase is likely attributable, in part, to enhanced detection (due to the popularity of the rapid UAT) and more complete surveillance and reporting. In the United States, the increasing median age, and concomitant decreasing health, of a growing population may also promote this trend (534, 558). However, the actual dis-

ease burden is likely underestimated, given that LD cases presenting as nondescript CAP (especially mild cases) may be treated empirically using broad-spectrum antibiotics that are active against *Legionella*, leaving the disease, and its transmission source(s), unrecognized and unreported. Consequently, LD-inclusive empirical treatment has contributed to the trend of declining mortality while masking both the true extent of disease and potential outbreak-related disease clusters (15, 535). When CAP is diagnosed and LD is suspected, laboratory confirmation is central to defining disease etiology, detecting unidentified LD outbreaks, and contributing to the larger goal of defining the scope of legionellosis in the United States. Education and awareness of LD by health care professionals, not as an exotic disease but as a potentially deadly contributor to community- and health care-associated pneumonia, are critical to decreasing mortality among at-risk populations. This is a key first step to understanding and addressing legionellosis in the coming decades.

An additional, unintended consequence of undetected outbreaks is the missed opportunity for further description of disease risk and clinical presentation. Most LD risk factors were originally compiled during outbreak investigations, and ongoing research is needed to fully document, refine, and continually update this description. For example, a wide spectrum of clinical features was recently reported for full-term infants exposed to *Legionella*-containing aerosols from a cold-mist humidifier (559). Surveillance has also demonstrated sustained, high levels of travel-associated LD cases, which creates challenges for health care systems in all countries and states, regardless of the regional LD prevalence (15, 202, 210). Federal and international surveillance networks, such as the National Notifiable Diseases Surveillance System (NNDSS) and the Waterborne Disease and Outbreak Surveillance System (WBDOSS) in the United States, are vital for recognizing these patterns and clusters of disease. However, monitoring networks are valuable only when health practitioners are familiar with the symptoms and risk factors for LD, leading to laboratory confirmation, rapid treatment, and timely reporting by state agencies.

### Controlling Disease Transmission

Legionellae are ubiquitous in natural ecosystems (both aquatic and terrestrial), and advances in building technologies, particularly since the 1950s (e.g., air-conditioning cooling towers, recreational water features, and complex water distribution systems), have provided ideal conditions for bacterial growth and persistence (6). High rates of morbidity and mortality may result when large human populations are brought into close proximity to respiratory pathogens, especially at locations that concentrate individuals in at-risk groups (e.g., transplant hospitals). Therefore, a second key to addressing legionellosis in the coming decades is minimizing or eliminating disease transmission through risk assessment, regular maintenance of potable and nonpotable water systems, and water monitoring and treatment in facilities that care for susceptible populations. Several informational resources are available to building managers, industrial hygienists, and administrators (as well as to clinicians, laboratorians, and the general public) to inform and guide their decisions in the design of a comprehensive plan to inhibit *Legionella* colonization or when contemplating action after a positive test result or disease is discovered. Links to the following resources are provided in Table 3. The American Society of Heating, Refrigerating, and Air-Conditioning Engineers (ASHRAE) is an international organization that provides standards, guidelines, and best practices to the building technology field. Numerous ASHRAE publications address the issues of legionellosis prevention and response in nonresidential locales, such as guideline 12-2000 and Standard 188P, which deal with minimizing and preventing LD associated with building water systems. The Environmental Protection Agency (EPA) published the *Legionella*: *Human Health Criteria* document, detailing bacterial ecology and distribution, with sections on risk assessment, analysis, and treatment. The WHO commits several chapters in its guide *Legionella and the Prevention of Legionellosis* to water safety plans (WSPs) as well as in-building distribution and exterior cooling system assessment; additional WHO documents discuss risk assessment for drinking water and water safety plans in buildings. The ECDC provides two *Legionella*-related Web resources, the Legionellosis Health Topic Web page and the European Legionnaires' Disease Surveillance Network (ELDSNet) website, which together include disease facts, recent surveillance reports and publications, *Legionella*-focused events, case definitions, operating procedures, and an outbreak investigation toolbox. Two United Kingdom agencies, the Health and Safety Executive and Public Health England (currently merging with the Health Protection Agency), also provide useful informational resources. The CDC *Morbidity and Mortality Weekly Report* (MMWR) publishes two related guidelines, the first document, *Guidelines for Preventing Healthcare-Associated Pneumonia*, addresses specific recommendations on the topic of *Legionella*, and the second document, coordinated by the CDC and the Healthcare Infection Control Practices Advisory Committee (HICPAC), is entitled *Guidelines for Environmental Infection Control in Health-Care Facilities* (560). In addition, the above-mentioned CDC *Legionella* Web page provides tools and protocols for environmental assessment, sample collection, and *Legionella* testing and a list of ELITE-certified commercial laboratories. Finally, there are many additional documents and resources from organizations and government agencies not included here that may serve as important guidance and instruction for interested parties. Table 3 is not meant to be an exhaustive list but rather representative of the types and scope of resources available.

The issue of ongoing microbial water monitoring is controversial and thus warrants special attention. The CDC is not currently positioned to unilaterally mandate regular assessment for *Legionella* in potable and nonpotable water systems. However, it is clear that institutions frequented by or housing susceptible individuals should be aware of and mitigate the LD risk for their occupants and visitors, as detailed in previous CDC guidelines (Table 3) (111, 560). Large numbers of individuals in these high-risk categories are concentrated in organ transplant units, intensive care units (ICUs), cancer centers, infant nurseries, and sites that commonly care for chronically ill or immunocompromised patients, among others. These facilities should be vigilant in clinical surveillance for *Legionella* and maintain a high index of suspicion for disease even when results of environmental testing are negative (561). Outside this institutional population, general hospitals and facilities for seniors and the elderly (e.g., LTCFs) should, at the least, undertake an infection control risk assessment and conduct ongoing LD surveillance, while cruise ship medical providers are recommended to employ rapid UATs in standard practice. These locales should also understand their water distribution systems and monitor temperature and levels of disinfectant (and/or water quality parameters) at distal point-of-use sites. The importance of

actively monitoring LD risk in non-health-care settings is less clear; this is not to say that other establishments shoulder little risk or bear no responsibility for the health of their patrons. As demonstrated by the original 1976 American Legion epidemic at a conference center hotel and the hundreds of LD clusters identified since then, complex water and air-handling systems require proper maintenance and disinfection regardless of their location or the typical clientele. An additional, distinct category is reserved for institutions that have experienced one or more LD outbreaks, especially in the past 5 years. Data from recently conducted CDC Epi-Aids suggest that disease transmission from a localized, potable water point source is typically symptomatic of wider, systemic plumbing network colonization (CDC, unpublished). In these instances, regular, short-term *Legionella* testing is advised, to monitor the success of remediation; yearly, long-term testing may also be warranted, to ensure that colonization does not recur.

### *Legionella* Persistence and Remediation

Of particular interest to facility managers in areas with a high environmental burden of *Legionella*, complex water distribution, or at-risk populations is the issue of *Legionella* persistence. The problem of long-term colonization in anthropogenic water systems has arisen many times in environmental assessments, retrospective studies (183, 562–565), and outbreak investigations (75, 92, 530, 566–571). As illustrated in one recent outbreak report (75), ~35 cases of LD in condominium residents were discovered over 9 years due to the mistaken belief that low-level potable water contamination did not pose a significant disease threat; seemingly arbitrary "action levels" for remediation laid out by facility managers and defined by bacterial concentration thresholds resulted in recurrent and prolonged transmission and disease. As data from the CDC ELITE program suggest, quantifying risk through such a strategy is problematic, because while most participating laboratories accurately determined the presence of bacteria (93% of samples correctly characterized), the precision in *Legionella* quantitation was very low (interlaboratory bacterial counts differed by up to 3 logs), with average 1.25-log underestimates of viable numbers (258). The difficulty and extreme variability in *Legionella* enumeration between different laboratories, sampling strategies, and culture methods, and even from the same source on different days, are reflected in previous reports as well (345, 561, 572, 573). Additionally, the numerical relationship between the colonization level and disease is at best complex and at worst misleading; for example, a recent metastudy evaluated an often cited metric for assessing LD risk that is based on an increased prevalence (≥30%) of hospital sampling sites being positive for *Legionella* (574, 575). Researchers identified 31 peer-reviewed journal articles representing 119 hospitals where reports of LD were temporally associated with environmental testing. The results indicate that the ≥30% positivity cutoff is neither sensitive (59%) nor specific (74%) for use in LD risk management within health care settings. While continued research is needed to confirm previous findings, at least two important points are clear: (i) there is currently no known safe concentration of *Legionella* in man-made potable and nonpotable water networks, which is due in part to unreliable bacterial enumeration in complex samples, potential day-to-day variability for *Legionella* detection at any individual source (258), and disease dependence on multiple individual-, environment-, and bacterium-specific factors (75), and (ii) risk assessment and environmental management must be a multilevel approach, based on proven science and best practices, and should account for complexities in system architecture, potential routes of exposure, and the susceptibility of present populations, among others. *Legionella* can persist for long periods under permissive control and monitoring policies. Quantification of risk based on arbitrary levels of detectable colonization (75, 574, 576) is currently a misinformed and ambiguous calculus at best; hence, the recommendation of unproven or incomplete approaches in response to positive environmental samples (577) is imprudent and ill-advised. Given these concerns, complete eradication of *Legionella* from man-made water and air-handling systems should be the stated goal, especially when cases of LD have been documented previously.

### Future Approaches for the Advancement of *Legionella* Diagnostics

Legionnaires' disease is an underappreciated, mostly sporadic illness. This poses a serious challenge and highlights three significant approaches that are key to addressing legionellosis in the coming decades; these approaches include (i) employing all available, reliable diagnostic tools (286); (ii) improving existing assays; and (iii) developing new technologies that offer increased sensitivity, specificity, availability, and/or efficiency. The diversity of specimen types and availability of laboratory test platforms enable (and warrant) multiple, simultaneous detection strategies. Toward this end, several groups have assessed the "added value" of a coordinated approach for the detection of *Legionella*. They found overall increases in diagnostic sensitivity and specificity by employing more than one complementary assay (e.g., a combination of PCR, culture, UAT, and/or IFA assay) (335, 344, 578).

Most current clinical diagnostics for LD were developed and commercialized in the 1980s and 1990s (http://www.fda.gov/). Among them, card- and ELISA-based UATs were widely adopted by clinical laboratories, leading to increased detection and reporting of LD but with a bias toward recognizing *L. pneumophila* sg1 infections. A dramatic decrease in the use of culture methods for *Legionella* identification was also observed in the same time period both in the United States and abroad. A combination of these trends has potentially led to the underrepresentation of LD caused by non-sg1 legionellae. As mentioned above, within the past 6 years, two additional FDA-cleared *Legionella* UATs were marketed in the United States, with sensitivities comparable to those of existing assays. Additionally, at least 5 apparently equivalent *Legionella* UATs were developed and sold abroad (Table 4). While different test formats (e.g., dipstick/card/lateral flow) may increase choice and help lower laboratory costs, no significant improvements in detection efficiency or test sensitivity have been realized for alternative *Legionella* serogroups or species. At least two in-house, validated, broad-spectrum *Legionella* EIAs have been described (579, 580), but the only promising development is the discovery of a genus-wide common immunodominant antigen in legionellae, peptidoglycan-associated lipoprotein (PAL) (581–584). To date, one company has applied research toward a potential pan-*Legionella* rapid ICT (SD Bioline *Legionella*) using this antigen. Despite an initial negative review, this ICT was compared favorably to the Alere BinaxNOW *Legionella* UAT for Lp1 diagnosis; however, further evaluation is needed to assess its potential for non-sg1 disease diagnosis (309, 585).

Neither FDA approval nor clearance is required for *in vitro* diagnostic tests developed and implemented within a clinical or ref-

**TABLE 4** Commercially marketed *Legionella* urinary antigen tests

| Company | Product name | Test format | FDA cleared | Commercial availability | Note |
|---|---|---|---|---|---|
| Alere | BinaxNOW *Legionella* urinary antigen card | ICT | Yes | U.S. | Approved only for Lp1 |
| Alere | Binax *Legionella* urinary antigen EIA | EIA/ELISA | Yes | U.S. | Approved only for Lp1 |
| Meridian Bioscience, Inc. | Tru *Legionella* | ICT | Yes | U.S. | Approved only for Lp1 |
| SA Scientific | SAS *Legionella* test | ICT | Yes | U.S. | Approved only for Lp1; based on >1 polyclonal antibody |
| Trinity Biotech | Bartels *Legionella* UAT | EIA/ELISA | Yes | U.S. | Approved only for Lp1 |
| Bio-Rad Medical Diagnostics GmbH[b] | *Legionella* urine antigen EIA | EIA/ELISA | No | Unknown | Reported to detect all *L. pneumophila* serogroups and some other species; however, this is unverified |
| ThermoFisher Scientific/Oxoid | Xpect *Legionella* UAT | ICT | No | Unknown | Reported to detect *L. pneumophila* sg1 and sg6 |
| Coris Bio | *Legionella* V-Test | ICT | No | Unknown | Reported to detect *L. pneumophila* sg1 |
| Trinity Biotech | Uni-Gold *Legionella* Urinary Antigen Plus | ICT | No | Unknown | Reported to detect *L. pneumophila* sg1 |
| Diamondial[b] | Rapid U *Legionella* Plus | Unknown | No | Unknown | |
| Standard Diagnostics[b] | SD Bioline *Legionella* | ICT | No | Unknown | Marketed for Lp1, based on pan-*Legionella* PAL antigen |

[a] ICT, immunochromatographic test; EIA, enzyme immunoassay; ELISA, enzyme-linked immunosorbent assay; UAT, urine antigen test; FDA, U.S. Food and Drug Administration.
[b] Very little information available.

erence laboratory (although other regulatory considerations apply, e.g., CLIA). However, not all laboratories have the time and personnel resources for in-house assay development. In the United States, commercially available *in vitro* diagnostic tests must have FDA clearance and therefore represent a prevalidated, "ready-to-use" system for the identification of disease-causing agents. Nucleic acid-based molecular diagnostics offer rapid, accurate results for CAP etiology and are available for many disease agents; unfortunately, only one *Legionella* species NAAT has been FDA cleared, but it is not commercially available. A simple, inexpensive, FDA-cleared NAAT for most disease-associated *Legionella* species, based on proven PCR or emerging technologies such as LAMP, would greatly empower health care providers and laboratorians who currently rely on tests of prohibitive length or cost or of limited specificity.

Since 2004, respiratory infections represented the largest human disease category worldwide and one of the leading causes of mortality (586). *Legionella* is among the top nonzoonotic atypical agents of severe respiratory illness, and successful disease resolution requires swift treatment together with rapid diagnosis for informed and accurate antibiotic management and epidemiological awareness. Traditional techniques, such as culture and serology, will continue to offer value for research, epidemiology, and typing purposes. Despite clear challenges, the future of *Legionella* and legionellosis detection in the 21st century is promising; novel molecular approaches increase sensitivity, ease of use, and efficiency, while existing assays are updated to recognize a wider spectrum of pathogens. Ultimately, all facets of *Legionella* research and education will lead to better surveillance, enable earlier disease diagnosis, and decrease the LD burden.

## ACKNOWLEDGMENTS

We thank Lauri Hicks and Claressa Lucas for their valuable insight and critical review of the manuscript and Meredith Boyter in the CDC Graphics Services Branch for her artistic eye and skill.

There are no disclosures or conflicts of interest to declare.

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