Molecular diagnostics of atypical pneumonia

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ABSTRACT

The emergence of nucleic acid-based molecular techniques has significantly enhanced laboratory diagnosis and monitoring of atypical pneumonia. These techniques have not only provided rapid and sensitive detection of fastidious microbial organisms but have also played critical roles in identifying and characterizing emerging pathogens that cause atypical pneumonia. Other benefits that molecular techniques can bring to the field include organism differentiation, quantitation, typing, and antibiotic resistance profiles. Gradually becoming standardized and widely available, the future will see some promising molecular methods become a mainstay in clinical laboratories for recognition and diagnosis of atypical pneumonia pathogens.

INTRODUCTION

The term and concept of atypical pneumonia arose in the early 1940s, when some cases of pneumonia did not respond to sulfonamides and then, penicillins[1]. This description can apply to diseases caused by a variety of bacterial, rickettsial, viral, fungal and even protozoan organisms (Tab 1). Despite the identification of multiple causes, atypical pneumonias share two unifying features. The first is a non-lobar patchy or interstitial pattern on chest radiography, and the other is a failure to identify a causative organism on Gram stain or sputum culture as routinely performed. Several atypical pneumonia pathogens caught the world's attention quite successfully by their extremely unusual "power", which included the first description of *Mycoplasma pneumoniae* atypical pneumonia syndrome in the mid 1940s[1], an outbreak of Legionnaires' disease in the 1970s[2], a *Chlamydia pneumoniae*-related atypical pneumonia mergence in the 1980s[3], a *Pneumocystis carinii* pneumonia identified in patients with AIDS in the 1980s[4], a human metapneumovirus causing respiratory tract disease in young children recognized in 2001[5], and a global outbreak of the notorious severe acute respiratory syndrome (SARS) earlier this year[6,7].

Clinical manifestations related to atypical pneumonia include fever, dyspnea, cough, and unilateral patchy segmental infiltrates, which are rarely organism-specific, especially in younger children. On the other hand, the etiological agent determines potential prognoses as well as optimal treatment modality for the patient suffering from atypical pneumoniae. SARS, characterized by its high mortality and contagiosity, does not respond to antibiotics (*eg*, macrolides), which are usually effective toward organisms commonly causing atypical pneumonia. Board range antibiotic therapy is a waste when atypical pneumonia is caused by viral pathogens. Therefore, early and accurate identification of the pathogenic organism causing the atypical pneumonia is critical to clinical intervention. When a life-threatening outbreak such as SARS occurs, the rapid identification will enable doctors to begin more a timely treatment of pa-

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Tab 1. An incomplete list of microbial organisms causing atypical pneumonia.

Category	Organism	Main diseases
Bacteria	*Bacillus anthracis*	Anthrax
	Bordetella species, including *B pertussis*, *B parapertussis*, and *B holmesii*	Whooping cough and others
	Brucella species	Brucellosis
	Burkholderia pseudomallei	Melioidosis
	Francisella tularensis	Tularemia
	Gram negative bacilli	Atypical (nosocomial) pneumonia
	Legionella pneumophila	Legionnaires' disease
	Leptospira interrogans	Leptospirosis
	Pasteurella multocida	Atypical pneumonia and others
	Yersinia pestis	Plague
Bacteria-like	*Chlamydia pneumoniae* (TWAR)	Atypical pneumonia
	Chlamydia trachomatis	Atypical pneumonia and others
	Chlamydia psittaci	Psittacosis
	Mycoplasma pneumoniae	Atypical (walking) pneumonia
Rickettsia	*Coxiella burnetii*	Acute Q fever
	Rickettsia rickettsiae	Rickettsiosis
Viruses	Adenoviruses	Atypical pneumonia and others
	Enterovirus	Atypical pneumonia and others
	Hantavirus	Hantavirus pulmonary syndrome
	Herpesvirus, including HSV, CMV, VZV, EBV, HHV-6, HHV-7, and HHV-8	Atypical pneumonia and others
	Influenza virus A, B, and C	Influenza
	Measles virus	Atypical pneumonia and others
	Metapeumoviruses	Atypical pneumonia and others
	Non-SARS coronavirus	Atypical pneumonia and others
	Parainfluenza virus 1, 2, and 3	Parainfluenza
	Respiratory syncytia virus	Atypical pneumonia and others
	Rhinoviruses	Atypical pneumonia and others
	SARS virus	SARS (atypical pneumonia)
Fungi	*Aspergillus species*	Aspergillosis and others
	Candida species	Candidosis and others
	Coccidioides immitis	Coccidioidomycosis
	Cryptococcus neoformans	Atypical pneumonia and others
	Histoplasma capsulatum	Histoplasmosis
Protozoa	*Pneumocystis carinii*	Atypical pneumonia
	Toxoplasma gondii	Toxoplasmosis

tients who have been exposed, and will more quickly alleviate undue anxiety for people who have not been exposed. Unfortunately, the microbial pathogens involved are sometimes difficult to identify and differentiate from large numbers and varieties of normal flora existing in the upper respiratory tract at the time the patient presents to the physician.

A microorganism from a sample collected from the respiratory tract can be detected and identified in any of four possible ways: (i) Cultivation of microorganisms using artificial media or living hosts, (ii) Direct microscopic examination or antigen detection, (iii) Measurement of microorganism-specific immune responses, and (iv) Detection of microorganism-specific nucleic acids. Conventional assays, including cultures and antigen and antibody detection, have not been satisfactory for the routine laboratory diagnosis of several atypical pneumonia caused by fastidious pathogens. For example, a specific laboratory diagnosis is seldom attempted for *C pneumoniae* because culture techniques are complicated, slow and generally available only in reference lab[8,9]. Although the culture method remains

the gold standard for the diagnosis of *Legionella infection*, its sensitivity is relatively poor[10]. Several methods developed for direct detection of *Legionella* species, including the gold standard culture, have suffered from their poor sensitivity[11]. The culture of *Coxiella burnetii* and the SARS virus must be done in biological safety level 3 or higher laboratories, which are not routinely accessible to most of clinical laboratories[12].

MOLECULAR TECHNIQUES

Technological revolutions in microbiology and molecular biology have significantly expanded and improved the capabilities of diagnostic microbiology. Molecular methods, replacing biological amplification by enzymatic amplification of specific nucleic acid sequences, has dramatically changed the way we detect and characterize infectious agents. These methods have not only enhanced diagnostic validity and decreased the turn-round time for patient results, but have increased clinical relevance of the information provided by the laboratory as well. As one technological milestone in biotechnology, PCR has simplified and accelerated the in vitro process of nucleic acid amplification and significantly broadened the microbiologists' diagnostic arsenal. Commercial kits and "home-brewed" procedures have been developed and applied to the detection of microbial path[9,12-15], the identification of clinical isolate[7,8,16], and strain subtyping[17-20] for physicians who take care of patients with atypical pneumonia. The detection and identification of amplification products, or amplicons, has become a routine procedure in the molecular diagnostic laboratory, which not only "visualize" the amplified DNA molecules but enhance test sensitivity and specificity. Such visualization techniques included classical agarose gel electrophoresis with or without a Southern blot hybridization[21], colorimetric microtiter plate system[14], direct sequencing[7,22], matrix hybridization[18], and recently developed "real time" system in which amplification and identification happen simultaneously[11,19,23,24].

MOLECULAR DIAGNOSIS OF ATYPICAL PNEUMONIA

Detection of unculturable, slow-growing or fastidious The rapid, in vitro enzymatic amplification characteristic of PCR indicates its primary application for the detection of organisms causing atypical pneumonia, which are usually unculturable, slow-growing or fastidious. Microbial nucleic acids extracted from a respiratory specimen may be analyzed for the presence of various organism-specific nucleic acid sequences regardless of the physiologic requirements or viability of the organism. For example, a sequence homology between the animal coronavirus and the newly identified SARS virus formed the basis to rapidly detect and identify the latter pathogen[7]. A colorimetric microtiter plate RT-PCR system was successfully used to detect and subtype respiratory syncytial virus (RSV) in nasal wash specimens[14]. It is an advantage for molecular techniques to have one universal multiplex procedure to detect human adenoviruses which contain at least 51 different serotypes[15]. A real-time RT-PCR test kit is available commercially for the rapid diagnosis of SARS virus-caused atypical pneumonia[6].

Laboratory monitoring of infections Many bacteria can exit in both a pathogenic and non-pathogenic state. Merely finding the organism, especially in the normal flora-colonized upper respiratory tract environment, does not imply that it is causing disease. In this scenario, molecular methods can be used to detect virulence determinants. Not all virulence determinants are chromosomally mediated, but molecular methods can be used to detect and identify these virulence factors carried by plasmids. An RT-PCR procedure was successfully applied to the differentiation of, for example, viable from non-viable *L pneumophila*[25] which is especially useful for chemotherapy efficacy monitoring. A PCR based test targeting *P carinii* in sputum samples from AIDS patients has been used to monitor treatment with pentamidine[26]. There has been growing demand for the quantitation of nucleic acid targets, which has been used to monitor therapeutic response and provide prognostic information. Quantitative detection of respiratory *C pneumoniae* infection was performed by a real-time PCR for the purpose of monitoring atypical pneumonia therapy[27]. Similarly, real-time RT-PCR assays were used to quantitate RSV and SARS virus RNA in nasal aspirate specimens[11,23].

Rapid identification of emerging pathogens Molecular methods have won superfluous credits regarding the discovery and characterization of novel pathogens causing atypical pneumonia. Within the past decade, PCR followed by a sequencing method successfully identified and characterized hantavirus, human metapneumovirus, and SARS viruses[5,7,22]. In addition to the detection of bacterial pathogens directly

from respiratory specimens[8,12,27], nucleotide sequence analysis of the small-subunit (16S) bacterial rRNA gene allows characterization of previously unrecognized bacterial species causing atypical pneumonia[16,28]. Since viruses lack ribosomal genes, several subtractive technologies allied to amplification methods have been used to identify novel viruses. Probably due to the "non-sterile" characteristic of respiratory tract specimens, these techniques have not been widely used to hunt for novel viruses causing atypical pneumonia.

Genotypic determination of antimicrobial resistance Antimicrobial susceptibility testing is one of the most important tasks in a clinical microbiology laboratory, which provides an *in vitro* estimate of the probability that an infection will respond to chemotherapy *in vivo*. Molecular techniques are starting to play a role in the rapid detection of resistance. In some cases, such techniques offer the opportunity to reduce the time required for the institution of definitive therapy, thus reducing the use of inappropriate antibiotics. Rapid detection may also allow early recognition of carriers infected by resistant organisms and the appropriate implementation of isolation, epidemiological investigation and integrated infection control practices. An RT-PCR-based method has been reported for antimicrobial susceptibility testing of *C trachomatis*[29]. The detection of a *tet*M gene by molecular methods has been used to determine tetracycline resistance in *Mycoplasma* species[30]. Molecular approaches have been used to detect influenza gene mutations related to reduced susceptibility to neuraminidase inhibitors and resistance to amantadine[25]. The emergence of erythromycin-resistant *B pertussis* has been traced to one mutation in the 23S rRNA gene, which can be detected by a PCR-based assay[31].

Epidemiology investigation enhancement Microorganism typing using molecular methods has important implications for the epidemiology investigation of atypical pneumonia. A bacterial restriction endonuclease analysis of bacterial chromosomal DNA was used to incriminate a water system as the source of a 32-case Legionnaires' disease outbreak[20]. Gene sequence analysis is the ultimate discriminatory tool, and a PCR followed by direct sequencing analysis was used to determine the possible epidemiologic relatedness between the SARS viruses recovered from humans and other wild animals[32]. A genetic analysis was used to type *B anthracis* isolates and trace the possible resource that resulted in the 2001 bioterrorism-associated anthrax outbreak in the US[17]. Real-time PCR and microarray assays have been applied for the typing and subtyping of influenza viruses directly in respiratory samples[18,19].

FUTURE DIRECTIONS

"Atypical pneumonia chip" Molecular screening of "at risk" populations for a group of possible and common pathogens causing atypical pneumonia is an exciting area. This idea is very important for quick identification and differentiation of various microbial pathogens, which is especially important for quickly alleviating undue societal anxiety. Traditionally, different methods of detection are employed for different groups of pathogens that can cause atypical pneumonia-like syndromes, which require special media, equipment, safety facilities, and expertise. Molecular techniques can screen a specimen for panels of probable pathogens. One of the PCR "cousins", multiplex PCR, utilizes numerous primers within a single reaction tube in order to amplify nucleic acid fragments from different targets[9,13,15,18]. Nucleic acids extracted from respiratory specimens of patients with atypical pneumonia are added into the multiplex PCR reactions. Specific nucleic acid amplification should occur if the appropriate target DNA is present in the sample tested. After PCR amplification, a special "AP chip", which includes an array of specific oligonucleotide probes, can be used to identify and type microorganism-specific PCR products[18].

Beyond bugs Enhanced by the human genome programs, clinical microbiology laboratories started to do something beyond microorganisms to help physicians manage infectious diseases. Polymorphisms in various alleles in several host immunogenetic factors have been described that influence the host immune response to infectious agents, thereby determining the host susceptibility to certain diseases and pathologic conditions. An unusual haplotypic structure of IL-8 is associated with host susceptibility to a common viral disease of infancy[33]. An association of severe RSV illness was demonstrated with IL-4 and its receptor polymorphisms[34]. If infections can be viewed as horizontally acquired genetic diseases, it makes perfect sense to view pathogen and host as an integrated system. Enhanced by the on-going human genome project, the detection of infection-related host gene polymorphism may become an increasingly important role in clinical laboratories in the future.

Tab 2. Diagnostic methods available for common agents causing atypical pneumonia.

Organism	Current test methods	Application of molecular tests	Comments	Selected references
Mycoplasma pneumoniae	Serology, culture and molecular	Detection, differentiation, and antimicrobial susceptibility	Serology is method of choice and molecular method is promising	(9, 24, 30)
Chlamydia pneumoniae	Serology, culture and molecular	Detection, differentiation, quantitation, and antimicrobial susceptibility	Molecular method is mostly promising	(8, 9)
Legionella pneumophila	Culture, antigen, and molecular	Detection, differentiation, monitoring, and typing	Culture is the gold standard and molecular method is promising	(9, 20, 25)
Bordetella pertussis	Culture, antigen, and molecular	Detection, differentiation, and antimicrobial susceptibility	Culture is the gold standard and molecular method is promising	(31)
Influenza and parainfluenza viruses	Culture, antigen, serology, and molecular	Detection, differentiation, antimicrobial susceptibility, and typing	Culture is the gold standard and both antigen and molecular methods are rapid	(13, 19, 21)
RSV	Culture, antigen, and molecular	Detection, differentiation, and quantitation	Culture is the gold standard and both antigen and molecular methods are rapid	(13, 14, 23)
Adenoviruses	Culture, serology, and molecular	Detection, differentiation, and typing	Culture is method of choice and molecular method is promising	(13, 15)
SARS viruses	Molecular, culture, serology	Detection and differentiation	Molecular method is method of choice and culture is only performed in higher biosafety levels	(6, 11)

Physician-laboratorian communication The exchange of relevant information between the clinician and the laboratory is essential for good patient care. During the time period to identify the pathogen causing atypical pneumonia, the laboratory would appreciate that physicians set their clinical "priorities", instead of blindly choosing from an available test menu. By knowing the initial, fragmentary results yielded in the laboratory, physicians would be better able to modify their clinical impression. Such a communication has been significantly facilitated by the development of the Internet, which has rapidly become an important source of medical information. Without electronic communication among health care workers, it would have taken years, instead of months, to reach the tremendous achievements in discovery and characterization of the pathogens causing SARS[35]. During the outbreak of SARS in Toronto, an electronic screening process was successfully developed to screen hospital personnel[36]. The widespread availability of computer-generated data interpretation of clinical laboratory determinations, new advances in technology, and the measurement of disease markers on a molecular basis have added a whole new dimension to the field of diagnostic microbiology.

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