Review paper

Diagnostics of atypical pulmonary infections

Diagnostyka atypowych zakażeń płuc

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Medical Studies/Studia Medyczne 2023; 39 (1): 65–72
DOI: https://doi.org/10.5114/ms.2023.126297

Key words: atypical pneumonia, diagnostic methods, *Mycoplasma pneumoniae, Chlamydia pneumoniae, Legionella pneumophila.*

Słowa kluczowe: atypowe zapalenie płuc, metody diagnostyczne, *Mycoplasma pneumoniae, Chlamydia pneumoniae, Legionella pneumophila*.

Abstract

Atypical community-acquired pneumonia (CAP) is a lung infection caused by atypical bacteria. It is associated with non-specific symptoms, the course of the disease is unusual and it poses a serious threat to patients. Even though CAP is quite prevalent, most cases remain undiagnosed, and clinicians rely solely on empirical therapy. The aim of this article is to characterize the most frequently used methods in diagnostics of atypical CAPs and evaluate their efficacy. A literature review showed that most of these techniques are still under development and there is a need for standardized diagnostic algorithms for atypical infections. Molecular panels and serological assays have been especially emphasized as they allow for rapid identification of etiologic agents and antibiotic resistance.

Streszczenie

Atypowe zapalenie płuc (AZP) to infekcja spowodowana przez bakterie atypowe. Charakteryzuje się nietypowym przebiegiem, mało specyficznymi objawami, a jej powikłania mogą nieść duże ryzyko dla pacjentów. Mimo że prewalencja tej choroby jest wysoka, duża część przypadków nie zostaje prawidłowo zdiagnozowana i klinicyści opierają się jedynie na terapii empirycznej. W artykule przedstawiono metody powszechnie stosowane w diagnostyce AZP wraz z krótką charakterystyką i porównanie ich skuteczności. Przegląd literatury wykazał, że większość technik wciąż jest w fazie rozwoju i nie posiadamy wystandaryzowanych algorytmów postępowania w przypadku atypowych infekcji. Szczególny nacisk został położony na panele molekularne i serologiczne, które pozwalają na szybką, skuteczną identyfikację czynnika etiologicznego oraz ocenę lekooporności.

Introduction

Much of the work of the clinician concerns the identification of microorganisms infecting the patient and instituting appropriate treatment. In microbiological diagnostic practice, many bacteria cause unusual symptoms of pneumonia and are known as "atypical". This term stems not only from the non-characteristic symptoms (e.g., not elevated temperature, exhaustion and cough) but also from the biological structure of the bacterial cell. In the past couple of centuries, "atypical" manifestations heralded the presence of "atypical" bacteria in the disease progression. Currently, advanced laboratory technologies allow better analysis of the influence of bacterial structure on the resulting condition. This review addresses the following bacteria: *Mycoplasma pneumoniae* (*Mp*), *Chlamydia pneumoniae* (*Cp*) and *Legionella pneumophila* (*Lp*). These were selected based on their frequent prevalence, yet obscure nature. The aim of this article is to summarize the knowledge on current diagnostic tools and provide an introduction to the diagnostics of atypical pneumonia infections for clinicians.

Mp lacks a cell wall, contains a small genome and has limited biosynthetic pathways – all of these make *Mp* hard to cultivate and diagnose. The pathogen has a destructive impact on the airway epithelium as it promotes apoptosis and ciliostasis [1]. *Mp* uses many pathogenic factors such as hydrogen peroxide and superoxides to colonize tissues. These chemicals induce internal stress in tissues and organs, leading to general failure or dysfunction [2].

Cp is a Gram-negative bacterium, producing a cell wall, but it does not contain peptidoglycan. This pathogen has two morphologically and functionally distinct forms: the elementary body (EB) which is

metabolically inactive and infectious, and the reticulate body (RB), which is a metabolically active form responsible for multiplication. The infection usually has a progressive course: because of the biphasic cycle of this bacterium, the infection first starts in the upper airways and it is followed by signs from the lower airways in about 1 to 3 weeks [3]. *Cp* causes chronic infections, while its reinfections may induce acute inflammation of the tissues.

Lp is a Gram-negative, intracellular bacterium. There are two distinct forms of its infection: fatal Legionnaires' disease (LD) and benign Pontiac fever. Also, it activates a robust inflammatory response through its interactions with alveolar macrophages.

As described above, atypical agents tend to differ from other well-known bacteria, due to differences in the structure of the cellular wall or its absence, as well as their small size, difficulty in cultivation, ability to survive intracellularly and induce a strong inflammatory response. These features make them difficult to diagnose and, coupled with *Legionella's* ability to produce β-lactamases, resistant to β-lactams, used as standard treatments for respiratory tract infections. This poses a serious challenge to clinicians. The clinical symptoms of infections caused by atypical bacteria do not have distinct characteristics and they may often resemble those presented by pneumococcal and other typical infections of the respiratory tract, meaning that they may easily mislead clinicians. The symptoms are related to the upper and lower airways and comprise rhinorrhoea, general malaise, sore throat, headache, hoarseness and non-productive cough. Fever is not always seen, and it usually does not exceed 38°C, apart from *Lp* infections [4]. However, there are specific symptoms that could be assigned to each pathogen. In the case of *Cp*, the symptoms such as coughing may persist for months after the bacteria have been eradicated. Rarely, the disease may lead to sinusitis, laryngitis, and otitis media [5]. Patients with *Mp* infection may suffer from chest discomfort with rales and wheezes [6]. *Mp* can also give symptoms not directly connected with the respiratory system, such as neurologic, cardiovascular, dermatological, digestive, hematological, and musculoskeletal disorders [7]. *Lp* infection may manifest as dyspnea, relative bradycardia and gastrointestinal problems, including nausea, vomiting and diarrhea. These symptoms are unique and can be used during the diagnostic process to identify infections caused by *Lp*. Pontiac fever is a non-specific disease caused by various species of *Legionella*, including *Lp*, and it is characterized by fever, headache, chills, myalgias, nausea, vomiting, and diarrhea. Happily, it is a self-limiting, febrile infection, ending usually within 9 days. Pontiac fever should be considered based on epidemiologic exposure. In contrast with *Lp* infection, such as legionellosis, no signs or symptoms of lower respiratory tract infection are present [8]. Extrapulmonary infection manifests as panniculitis, possible myositis, and myocarditis in the absence of pneumonia, but it could be caused by species of *Legionella* other than *Lp*. Some of these, including cutaneous and subcutaneous *Legionella* infections, are rare and mostly occur in immunosuppressed patients [9]. Generally, infections caused by *Lp*, *Mp* and *Cp* usually have a mild course, whereas most *Lp* infections, such as legionellosis, are treated in the Intensive Care Unit. *Cp* and *Mp* are most often associated with muscle pain, weakness, and dry cough, while cases of *Lp* infections may lead to an acute phase with extrapulmonary symptoms. It is worth remembering that cases should be considered individually, because clinical symptoms do not follow one specific scheme of disease progression.

Epidemiology

A 2016 meta-analysis found the worldwide prevalence of atypical pathogens in community-acquired pneumonia (CAPs) to be 10.1%, 2.7% and 3.5% for *M. pneumoniae*, *L. pneumophila* and *C. pneumoniae*, respectively [10]. The factors associated with atypical CAP are younger age, female sex, and fewer comorbidities, and it is most often found in ambulatory or outpatient settings. A few problems emerge from these statistical analyses: patients with CAPs are not routinely tested for the presence of atypical pathogens (in particular, severe cases of CAPs are left undiagnosed), these tests are not standardized between different countries and conventional diagnostic tools are not proficient in cases of atypical bacteria [11].

Diagnostics

Mycoplasma pneumoniae

Mp is the most frequent bacterium causing CAP so it has been given the primary spot. It is of great significance to patients with asthma and any respiratory inflammations, such as viral infections caused by human rhinovirus (HRV), as they are more vulnerable to CAP [12, 13].

The "six factors classification", also known as the Japanese Respiratory Society (JRS) "diagnostic test", can be used by clinicians to improve the process of identification of *Mp*. The JRS test consists of six characteristics to describe the patient: 1) age under 60, 2) no or minor underlying disease, 3) stubborn cough, 4) poor chest auscultatory findings, 5) no sputum or etiological agent identified by rapid diagnosis, 6) a peripheral white blood cell count (WBC) less than 10 000/μl. When more than four of these criteria are positive, atypical pneumonia is suspected. Due to the biological characteristics of *Mp*, such as the difficulties in culturing and specific circumstances under which these microorganisms need to be multiplied, commercially used methods are not very effective for diagnosis. Commonly used methods include identi-

fication of the presence of cold agglutinins (cold agglutin test – CAT), enzyme-linked immunosorbent assay (ELISA), serological methods such as particle agglutination (PA), and complement fixation (CF). According to Wijesooriya *et al.* (2016), CAT is a less reliable method of tracking *Mp* pneumonia than isotype-specific ELISA kits (IBL – Hamburg Company, Germany) which detect immunoglobulin G (IgG) and immunoglobulin M (IgM) antibodies and allow calculation of seroconversion of IgG, because of its positive predictive values and lower sensitivity [14]. In the case of culturing *Mp*, it is not uncommon for growth in culture to take at least 3 to 4 weeks, which is too long for diagnosis of a disease. In some studies, it is claimed that bacterial growth can be hastened by changing the culture medium, such as by adding catalase [15]. However, a better method would be polymerase chain reaction (PCR) typing, which can help to identify the particular species of *Mycoplasma*, even if the number of cells is not tremendous. Using serological methods, IgM antibodies can be detected about 1 week after the presentation of symptoms and IgG after 2 weeks. Their levels can reach extremes during the third and fourth week of illness. Moreover, the measurement of IgG levels in a single acute-phase serum specimen can lead to uncertain results, because some individuals have high levels of these antibodies throughout their whole life. For this reason, diagnosis of IgG and IgM has rather retrospective value. The standard in diagnostics that can confirm the presence of *Mp* is a 4-fold rise in antibody titer. Also, the confirmation requires two serum samples, taken 2 weeks apart, positive for both IgM and IgG [16].

In the past couple of years, rapid diagnosis of *Mp* has been the subject of many studies, but the method most useful in the clinical setting is yet to be found. So far, it has been proven that community-acquired respiratory distress syndrome (CARDS) toxins have high diagnostic value. They may be detected in serum with ELISA and be used to confirm cases of *Mp* infections. However, loop-mediated isothermal amplification (LAMP) seems to be a faster and cheaper test to detect *Mp* at the bedside [17, 18]. In addition, immunochromatographic assay using colloidal gold shows almost 100% sensitivity and specificity [19], and Ribotest Mycoplasma (another immunochromatographic assay which detects the ribosomal protein L7/L12) is claimed to yield positive results which are highly prognostic in *Mp* culture-positive infection; however, false-negative results are obtained in one-third of samples containing *Mp* [20]. Both abovementioned methods exceed those with the use of amplification, mainly due to the significantly shortened time to obtain the result. However, if the diagnosis using these rapid methods is ambiguous, multiplex PCR seems to be an adequate method for recognizing *Mycoplasma* species [21].

Positive results from serology and PCR can be confirmed by diagnostic imaging. A study conducted in children in 2018 found computed tomography (CT) to be successful in 94% cases of positively *Mp* claimed patients. These radiological findings include hilar adenopathy, lobar infiltration, atelectasis and pleural effusions [22]. A study conducted in Japan found consolidation in chest X-ray to be a major radiological finding in both children and adults [23]. The process of diagnosis can also involve CT and, according to a study conducted by Huo *et al.*, the differentiation between coronavirus disease 2019 (COVID-19) and *Mp* infection can be seen [24]. COVID-19 gives the so-called crazy-paving signs (thickened interlobular septa and/or intralobular lines imposed on diffuse ground-glass attenuation) in the dorsal outer zone of the lungs and *Mp* gives fog signs along the bronchi [24]. In conclusion, CT and X-ray imaging can be used as a support during the process of diagnosis, but neither should be used alone.

Mp infection can also be confirmed by blood testing. Although this method is not directly used in diagnosis, clinicians report significant changes in blood composition during infection. Especially, in patients co-infected with Sars-CoV-2 and *Mp*, several blood parameters were increased. These were: blood urea nitrogen (BUN), creatinine, troponin, and fibrinogen, as well as inflammatory markers, including interleukine-6, C-reactive protein (CRP), erythrocyte sedimentation rate (ESR), serum ferritin, lactate dehydrogenase (LDH) and D-dimer [25]. Previous findings also confirmed that WBC count, CRP levels and lymphocyte count were elevated, exceeding the normal range [26].

Mycoplasmosis is said to be a generally self-limiting disease, and so antibiotic treatment is generally not needed; however, in serious cases, the disease progression will result in the usage of drugs.

Therefore, in patients who have multidrug resistant *Mp* (MRMP), tetracyclines and fluoroquinolones are going to be used as second-line antibiotics. The results of studies conducted by Kawai *et al.* and Wu *et al.* even indicate that fluoroquinolones are more effective in the therapy of *Mp* [27, 28]. US Food and Drug Administration (FDA) guidelines state that fluoroquinolones can only be prescribed to patients with complicated infections and for whom there is no suitable alternative antibiotic [29]. Tetracyclines can also be more effective in the treatment of macrolide resistant *Mp*, but there are several side effects of their usage [30].

Legionella pneumophila

There are almost 58 species and 80 serogroups in the *Legionella* genus, but the infections are mainly caused by the *Lp* serogroup 1 [31]. It is more likely to cause disease in people with a history of smoking or chronic diseases (especially of lungs), and those

older than 60 years or who may be immune compromised. Faradonbeh *et al*. indicate that sex has only a very small influence on morbidity, with men being infected a little more often than women [32]. Drinking water from unknown and uncertified sources is also a risk factor, because *Lp* dwells in watery habitats and hot-water systems made by humans. It is known that the spread of *Lp* has a greater impact on people in closed communities, such as military bases, package tours and tribes.

CRP and blood sodium levels may be good diagnostic predictors of *Legionella* CAP, as well as a high level of LDH and high body temperature [33]. Radiographic findings are various and nonspecific; however, the most common findings are patchy unilobar infiltrates, which can progress to consolidations. It has been found that during early radiographic and tomographic imaging, no significant difference in the chest CT scan is visible between patients with *Lp* and those with community-acquired pneumonia of other bacterial origin [34].

More specific diagnostic techniques include serology, urinary antigen testing (UAT) and molecular approaches. Serology-based methods are not advised by the Centers for Disease Control and Prevention, because 20–30% of patients with confirmed LD do not ever seroconvert and *Legionella* antigens are found in at least 20% of healthy, adult patients [35, 36]. To confirm LD, a four-fold increase in antibodies is required. Samples should be collected 2 weeks after the onset of symptoms and then 3 to 6 weeks later. The most frequent methods include the indirect fluorescence test (IFA) and ELISA. Two significant disadvantages of serological methods are that they do not allow differentiation between serogroups, and they may be prone to cross-reactions among the *Legionella* family [37]. Currently, the most widely used method is UAT, and it has replaced serological testing in routine diagnostics. Even though it can only detect serogroup 1 (and serogroup 6 in some cases), it still covers about 90% of all *Lp*. infections, and it offers low price, rapid results and good reliability [38, 39]. Obtaining samples is not problematic; therefore it is recommended for cases of severe pneumonia. UAT is available in the European Union in the form of commercial kits and is based either on enzyme immunoassay (quantitative) or immunochromatography (qualitative). It is worth noting that unlike cultivation, UAT is not affected by antibiotics, so it may be used freely during the therapy [35]. Molecular methods for diagnosing *Legionella* CAP involve conventional PCR, reverse transcriptase PCR, multiplex PCR and isothermal amplification.

Induced sputum has a higher yield for detection than nasopharyngeal aspirates and throat swabs [40]; however, acute cases of legionellosis are often associated with non-productive cough, and so it may be problematic to obtain samples. Research shows that PCR has a specificity close to 100%, but it cannot be used in retrospective research and the process of obtaining, storing and transporting specimens must be standardized to avoid any factors decreasing PCR quality [41]. Currently, there are no particular genes or markers for the detection of *Lp*. Diagnosticians typically search for the mcr gene; however, ssrA and wzm have also been proposed as candidates [35].

Other, less conventional methods include direct fluorescent antibody (DFA), slide agglutination tests and monoclonal antibody-based dot-blotting (MAb blot). These methods allow for typing at species and serotype levels, and qualitative identification. Meta-analyses show that DFA has rather low sensitivity (60%) and high specificity, so its performance is comparable with cultivation [41]. Despite this, the technique is rapid, with results being obtained in three to 4 h, and allows the detection of multiple *Legionella* serotypes; however, it may be prone to cross-reactions if polyclonal antibodies are used and its reagents are not at the disposal of all laboratories, meaning that its results should be treated as supportive evidence only [39].

It is widely accepted that the first-line antibiotics for legionellosis include quinolones and macrolides. Several papers have shown that there is no significant difference in the effectiveness of the two groups; however, quinolones are associated with fewer complications [42, 43]. Recently, it has been suggested that patients with *Lp* infection may be treated with a single dose of azithromycin, applied intravenously. This approach provides comparable medical outcomes with standard antibiotic treatment, while avoiding certain risks associated with prolonged antibiotic usage [44].

Chlamydia pneumoniae

The diagnosis of *Cp* remains troublesome due to its biology. This intracellular pathogen causes chronic infections with very few symptoms; as such, clinicians should rely mainly on serological and molecular methods. *Cp* infections are associated with male gender, age over 60 years, smoking habits and lack of spare time physical activity [45]. It has also been found that even though *Cp* is found mainly in elderly patients, it is the main cause of atypical CAP in children and adolescents aged from 5 to 20 years old. Also, *Cp* is connected with chronic infections, and antibodies against its antigens are found in about 80% of the general population. This makes differentiating between chronic infections and acute cases particularly problematic.

The diagnostic process may be assisted by imaging tests which reveal quite specific changes in the airways. X-ray may show consolidation shadows in both lungs, centrilobular nodules or tree-in-bud patterns, ground-glass opacity, alveolar infiltrate and air bron-

chogram. Further testing with CT may also reveal bronchovascular bundle thickening, emphysema or airway dilation [46, 47]. It has been suggested that pulmonary ultrasonography may be used to confirm cases of atypical pneumonia, as it shows B lines with coarse and thickened pleural line points [48]. Laboratory findings may be misleading, as they often do not show any abnormalities. However, in some cases chlamydial infection is associated with elevated levels of CRP and aspartate aminotransferase. WBC count remains on a normal level or slightly decreased [49, 50]. Recent research has also proposed a few novel biomarkers of chronic *Cp* infections, such as interferon γ (IFN-γ) and CD4+ effector memory T-cells (TEM cells), bacterial nucleic acids in mucus samples and bacterial lipopolysaccharide (LPS); however, these require technically demanding methods [51]. It is impossible to culture *Chlamydia* using standard methods as it is an intracellular pathogen, so cell cultivation is required. Still, it takes 2 to 3 weeks to obtain the results; cultivation is technically demanding and is associated with unacceptably low sensitivity. Some reports even indicate that confirmation of positive *Cp* cases is not achievable in a standard laboratory setting [50]. Therefore, this method is used solely in research facilities. Immunohistochemical (IHC) methods are not favored because of the nature of the bacteria: they are found in the deeper layers of epithelium, so more aggressive sample collection would be necessary and the amount of ER/RB may be too low for detection.

Currently, in epidemiological investigation and detection of acute infections, the most widely used method in the microbiological diagnosis of atypical CAP is serology. It is considerably cheap, fast and technically undemanding. Unfortunately, *Cp* pneumonias pose a serious challenge due to the fact that both IgM and IgG antibodies are produced with a significant delay, so the timing of collection of specimens is crucial [52]. Another obstacle is the high prevalence of *Cp* – it is suspected that *Cp* specific antibodies may be found in 50–80% of the population. Currently, laboratories offer a few options; however, the microimmunofluorescence test (MIF) is considered to be the gold standard of diagnosis, despite its several limitations. MIF uses purified elementary bodies to detect *Cp* antibodies. Interpretation of the results requires expertise and the endpoints are subjective, which restricts the possibility of regulating MIF titers and standardizing it between different laboratories. What is more, this test is retrospective, due to the necessity of obtaining paired serum samples and it is of rather low specificity, thus creating the possibility of invalid diagnosis [53, 54]. Its disadvantages may be overcome with ELISA, especially its multi-protein subtype. This method excludes one major difficulty, that is the cross-reactivity of chlamydial proteins. Commercial ELISA antigens have been found to be insufficient in distinguishing between *Chlamydia* subspecies; therefore, combining multiple peptide antigens in anti-*Cp* IgG ELISA is recommended [52]. It is worth noting that certain ELISA assays are also reactive among *Mp* positive patients.

Immunoblotting is another technique that may be used to confirm the diagnosis, and it has a few advantages. Firstly, it allows for the simultaneous detection of multiple antigens, giving high sensitivity. Secondly, it may be used to differentiate between various immunoprofiles. Lastly, comparative studies show that immunoblotting has a similar or even better specificity and sensitivity than MIF [55]. One important point is that surface proteins of *Cp* are not diagnostically reliable [56].

To promote rapid and easy testing, serologic panels have been developed, allowing for detection of anti-*Chlamydia pneumoniae*-specific antibodies IgA, IgG and IgM. Four are available in Europe: Hitazyme-ELISA, ELNAS Plate, LabSystems EIA and recombinant enzyme immunoassay [57]. Even though these tests show positive correlation with MIF, they are prone to false-positive results, so clinicians should approach the results with caution. It seems that serologic panels are most useful as screening tests in the case of chlamydial outbreaks; however, more sensitive techniques are preferred in non-epidemiologic scenarios [58]. Molecular methods surpass serology in one crucial aspect, which is the time to diagnosis: classic serologic methods require 1 to 3 weeks of delay in order to obtain paired serum samples and so they cannot be used to confirm a case and institute therapy ad hoc. Amplification methods may detect the pathogen rapidly, based on a small sample of mucus, laryngeal swabs or induced sputum. Also, PCR is associated with far better specificity (up to 95%); thus it excludes the possibility of cross-reactions, has a high throughput and it enables diagnosticians to establish objective cutoff points [59]. On the other hand, amplification methods are susceptible to contamination and do not allow one to properly differentiate ongoing infections from colonizations. While real-time PCR is the recommended method, multiplex PCR seems to be more practical in a clinical setting, as it allows for simultaneous detection of several CAP-related bacteria, and it is available in the form of commercial kits, which may be freely used by patients in ambulatory settings.

Cp (and other bacteria from the *Chlamydia* genus) is susceptible to antibiotic agents which interfere with deoxyribonucleic acid (DNA) and protein synthesis, such as tetracyclines, rifamycins, quinolones, macrolides and clindamycin, and it is naturally resistant to sulfonamides, glycopeptides and aminoglycosides [60]. *Cp*-related pneumonia should be treated with azithromycin, tetracycline, doxycycline or fluoroquinolones [61].

CAP has a complex clinical diagnosis due to the fact that its manifestations are not specific, it can be caused by a great number of pathogens and can frequently

have a mixed etiology. Hence, increasing attention is being paid to the development of multipathogen detection systems based on multiplex PCR. For example, BioFire FilmArray Pneumonia Panel (BioMerieux, France) is an FDA approved and CE-marked assay for quantitative and qualitative diagnosis of 34 infectious agents, including *Mp*, *Cp* and *Lp*. Multicenter studies have confirmed its over 95% sensitivity and specificity for most pathogens, both for bronchoalveolar lavage and sputum specimens [62]. This assay also allows several genes of drug resistance to be tested, although this does not apply to atypical bacteria.

Another option is the Curetis Unyvero P50 Pneumoniae Panel (Germany); it is CE-marked, and detects many pathogens, including all those described, together with resistance genes. Its sensitivity ranges from 50% to 100% and specificity is about 90%. Although these values are lower than those of BioFire FilmArray, it is still more effective than cultivation [63–65]. The third assay, Multiplex Lightmix RT-PCR (TIB MOLBIOL GmbH, Germany), appears to be just as accurate as singleplex RT-PCR, and it is capable of detecting various *Legionella* serotypes [66]. However, numerous alternatives are available in Europe.

These detection systems are also faster, as results are obtained within a few hours, they demand minimal technical expertise and they are not affected by antibiotic therapy. The further development and distribution of automated assays may greatly facilitate the therapeutic management of patients with severe pneumonia, thus reducing mortality and preventing significant complications.

Conclusions

Atypical infections still pose a serious problem to clinicians and their patients. In order to make a proper diagnosis and implement direct therapy, a multidisciplinary approach should be taken. As it has been shown, atypical bacteria generally do not induce strong changes in the infected organism. The clinical picture, based on standard diagnostic methods, may be misleading. Therefore, a strong emphasis should be placed on molecular panels. Automated assays quicken the diagnostic process, allow the differentiation between different atypical agents, which may be of great significance in cases of acute diseases, and they also make it possible to search for drug-resistance genes. Summing up, these assays provide the diagnosis together with advice on further treatment, which may shorten the length of therapy, reduce its costs and prevent complications.

An exemplary diagnostic process includes: 1) subjective symptoms and risk factors – unspecific signs, but bizarre manifestations such as extrapulmonary symptoms may guide clinicians to suspect atypical etiology; 2) laboratory analysis – increased acute-phase proteins and other, non-standard findings; 3) imaging – changes in both lungs and along the airways, such as lobar infiltrations, consolidation shadows, airway dilation; in the case of *Cp* ultrasound may also be useful; 4) specific methods – cold-agglutinin testing for *Mp* and urinary antigen testing for *Lp*; 5) serology – 4-fold rise in both IgM and IgG in two samples collected over 2 weeks (more useful in retrospective studies); 6) amplification methods – great sensitivity and specificity, but they are not available in every clinic. Multiplex PCR and isothermal PCR are more versatile as they can be used to search for several pathogens in a sample at once; 7) molecular panels – quick, efficient and very versatile. These can be used to make a proper diagnosis and determine drug resistance genes of some pathogens; 8) culture – appears to have very low value in a standard clinical setting; 9) other – MIF, blotting and slide agglutination are great for confirming cases in retrospective studies.

Conflict of interest

The authors declare no conflict of interest.

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