SHORT REVIEW

Laboratory diagnosis of *Streptococcus pneumoniae* infections: past and future

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Key words

Cultures • PCR • MALDI TOF

Summary

Streptococcus pneumoniae is one of the most important causative agent of pneumonia, meningitis, bacteremia, sinusitis and otitis media. The gold standard diagnostic method is still culture even if bacteriological diagnosis is making progress in molecular biology and in proteomics areas.

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Streptococcus pneumoniae (pneumococcus) is gram-positive, α-hemolytic, aerobic, encapsulated diplococcus. It is one of the most important causative agent of pneumonia, meningitis, bacteremia, sinusitis, and otitis media. In 2005, WHO estimated that 1.6 million people die of pneumococcal disease every year; 0.7-1 million of them are children aged < 5 years, most of whom live in developing countries. In the developed world, children aged < 2 years and elderly people carry the major burden of disease. Clinical conditions associated with immune deficiency increase the likelihood of contracting pneumococcal disease. Moreover the growing resistance of *Streptococcus pneumoniae* to commonly used antibiotics underlines the urgent need for vaccines to be used to control pneumococcal disease.

Pneumococcus may be harbored in the upper respiratory tract particularly in children with a rate of colonization of about 60% in children in winter and early spring, and 2% in adults.

The clinical signs and symptoms of pneumococcal infections cannot be differentiated from a disease of alternative etiology so the diagnosis of *Streptococcus pneumoniae* infection is frequently a problem.

The "gold standard" diagnostic method is still culture, but good-quality samples are not always available. Furthermore, cultures are not infrequently negative in infections considered likely on clinical grounds to be of pneumococcal origin, particularly after antibiotic administration [1, 2].

The laboratory diagnosis of pneumococcal infections currently is based on methods already used for many decades. The first step of investigation is the gram-stained smears of clinical specimens. Gram-positive cocci arranged as pairs of lanceolate-shaped cells can be observed.

Specimens that may be expected to yield pneumococci on culture should be plated onto a sheep blood agar or chocolate agar. After overnight incubation at 35° C with 5% CO2, *Streptococcus pneumoniae* shows a spectrum of colony types related to the degree of encapsulation generally surrounded by a large zone of green alpha-hemolysis, caused by a partial lysis of the erythrocytes. Heavily encapsulated strains develop colonies several millimeters in diameter, mucoid, gray, similar to drops of oil on the agar surface. Less encapsulated strains appear smaller. On prolonged incubation the central portion of the colony may collapse giving the characteristic "checker piece" appearance. Susceptibility to optochin (ethylhydrocupreine) and bile solubility are used to differentiate *Streptococcus pneumoniae* from the other viridans streptococci. Optochin is an antibacterial agent not used in therapy but only for the differentiation of streptococci. Usually pneumococci are optochin susceptible though optochin resistant strains are described [3]. The bile solubility test, based on the autolysis of *Streptococcus pneumoniae* in the presence of the surfactant sodium deoxycholate, is considered as being very sensitive and specific for identification of pneumococci.

The Quellung reaction is a more specific method for pneumococcal detection from pure cultures or sputum samples. After reaction of the pneumococcus with streptococcal anticapsular antisera, the pneumococcal capsule becomes visually enhanced, and the bacterial cell appears to be surrounded by a halo. While this reaction is considered specific for pneumococcus, cross reaction reported with other streptococcal polysaccharides will produce false negative results [4, 5]. The laboratory investigation must take into account the site of infection: respiratory tract, middle ear, central nerv-

ous system, eye, blood, bones and joints are sites where pneumococcus can be potentially associated with the disease.

The isolation of *Streptococcus pneumoniae* from blood culture define diagnosis of pneumococcal disease however rates of positive blood culture results for adults hospitalized with pneumonia are only 3%-8% [6] and are lower in children [7]. Bacteremia in pneumococcal meningitis occurs more frequently than in pneumonia (> 50%) [8].

Low density of the microorganism in blood, prior administration of antimicrobials, the intermittent bloodstream invasion, the bacterial autolysin released during the stationary growth phase, contamination of blood samples and cultures, insufficient volume, incorrect ratios of blood to broth, delays in transporting blood culture bottles to the laboratory, delays in performing subcultures of blood cultures, explain why the traditional methods of bacterial growth on media often fail [9]. Nevertheless the expert group for the diagnosis of pneumococcal pneumonia recommend that blood culture should be used, when available, because it can provide a specific aetiological diagnosis. In this situation the diagnosis of pneumococcal pneumonia is challenging. The utility of sputum Gram stain and culture is variable and is influenced by the ability of the patient to produce a good specimen, laboratory screening practices, and administration of antimicrobial drugs before the collection of specimens. On the other hand ideal diagnostic tests should not necessarily rely on the culture of viable organisms, must have high specificity and sensitivity, and should not be affected by prior antibiotic treatment. In addition, they should be inexpensive and easy to use without extensive training. In this context the development of an immunochromatographic test that detects the C polysaccharide cell wall antigen, common to all strains of *Streptococcus pneumoniae*, in urine (BinaxNOW, Binax, Portland, Maine), have improved the rapid diagnostic tools. It has a sensitivity of 70%-80% and a specificity of > 90%, compared with conventional diagnostic methods for detection of pneumococcal pneumonia in adults [10-12].

However the assay lacks specificity in children, because it can give positive results in healthy children with carriage of pneumococci and of other closely related Streptococcus species [13].

This assay has been successfully used with specimens of other body fluids, particularly for the rapid diagnosis of pneumococcal meningitis with use of CSF samples, with a sensitivity of 95%-100% and a specificity of 100% [14]. It has also been successfully used with pleural fluid specimens obtained from children and adults with pneumonia [15]. New assays based on the detection of other pneumococcal targets in urine samples are studied. For example the putative proteinase maturation protein A (PpmA) of *Streptococcus pneumoniae* is homologous to members of the family of peptidyl-prolyl cis–trans isomerases (PPIases), which accelerate the rate-limiting cis-trans or trans-cis conformational changes at X-Pro bonds during protein folding. The protein is a surface-located immunogenic lipoprotein that contributes to bacterial virulence. In literature is reported that the kidney barrier in patients with pneumococcal infection is permeable to the *Streptococcus pneumoniae* protein PpmA, resulting in its excretion in urine, which can be detected. At the moment more studies are required to be validated [16].

Nucleic acid amplification tests, such as PCR, are important diagnostic tools. They can detect small amounts of nucleic acid from potentially all pathogens, they do not depend on the viability of the target microbe, they are not affected by prior antimicrobial therapy and they provide results within a short time frame. LytA (autolysin) and Pneumolysin gene (ply) are genes target more used. LytA PCR has been shown to be more sensitive than detection of polysaccharide in urine [17], while Ply PCR is not specific and shows cross-reactions with other streptococcal species. The gene target is important, but the segment of the gene targeted is more important, owing to allelic variation in the target gene between closely related species [18].

In the etiologic diagnosis of pneumonia, PCR has a sensitivity for detecting *Streptococcus pneumoniae* in blood samples ranging from 29% to 100% [19]. The limits of molecular tests in blood samples may be explained by the rapid clearance of the *Streptococcus pneumoniae* from the blood stream and by sampling errors. Moreover positive pneumococcal PCR results have also been recorded from asymptomatic subjects [20] and these findings could be related to temporary bacteremia.

Regarding sputum samples, reported PCR positivity rates have ranged from 68% to 100% for samples from patients with pneumonia [19], although the colonization of the upper respiratory tract could affect the results. Moreover the presence of the pneumolysin gene in some nonpneumococcal viridans streptococci represents a problem of cross-reactivity [21]. Unlike for pneumococcal pneumonia, detection of pneumococcal DNA in CSF specimens can be useful for diagnosis of pneumococcal meningitis with high sensitivity and specificity (92%-100% and 100%, respectively) [22].

Matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) is starting to be used in clinical microbiology laboratories for routine bacterial identification from colonies.

Mass spectrometry (MS), a rapid, powerful, and sensitive analytical tool has been used recently for the differentiation, identification, and characterization of microbial pathogens. In particular, MALDI-TOF have been used to analyze whole bacterial cells that have not been modified chemically or by mechanical disruption [23]. By use of this technique protein/peptide fingerprints can be generated based on a proteomic profile and these patterns could serve as protein biomarkers that may prove useful for diagnostic purposes. Pro-

teomic profiling is an alternative to biochemical and genome-based identification schemes. MALDI-TOF MS has also been used for diagnosis on positive blood cultures. This technology is promising, as it reduces the cost and time to result of bacterial identification. However, the performance of MALDI-TOF MS for Streptococcus spp. is generally weak, and further studies are required to improve differentiation between *Streptococcus pneumoniae* and closely related viridans species such as Streptococcus mitis [24, 25].

Conclusions

Medical bacteriological investigation is based on five steps: inoculation of the specimen, incubation, reading the plates, identification and susceptibility testing. The question is how well these steps have been performed and how they can be improved for optimal identification and characterization of the major respiratory pathogens. New technologies for specimen processing, marker detection and typing (mass spectrometry, microarrays, and sequencing) could facilitate the implementation of diagnostics in laboratories. However the use of a combination of assays in parallel must be carefully evaluated, including cost-effectiveness aspects. PCR is the best known and most implemented diagnostic molecular technology to date. It can detect specific infectious agents and determine their virulence and antimicrobial genotypes with greater speed, sensitivity and specificity than conventional microbiology methods. Bacteriological diagnosis is continually advancing, particularly in the areas of proteomics and genomics. Matrix-assisted laser desorption ionization-time-of-flight (MALDI-TOF) and electrospray ionization (ESI) mass spectrometry (MS) and tandem mass spectrometry (MS-MS) have been at the core of these developments even if further studies are required to improve the differentiation between *Streptococcus pneumoniae* and related viridans species.

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■ Received on January 30, 2012. Accepted on February 28, 2012.

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