DE GRUYTER Eur. J. Nanomed. 2016; 8(3): 115–128

Critical Review

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Improving diagnosis of pneumococcal disease by multiparameter testing and micro/nanotechnologies

DOI 10.1515/ejnm-2016-0012
Received April 25, 2016; accepted May 12, 2016; previously published online June 3, 2016

Abstract: The diagnosis and management of pneumococcal disease remains challenging, in particular in children who often are asymptomatic carriers, and in low-income countries with a high morbidity and mortality from febrile illnesses where the broad range of bacterial, viral and parasitic cases are in contrast to limited, diagnostic resources. Integration of multiple markers into a single, rapid test is desirable in such situations. Likewise, the development of multiparameter tests for relevant arrays of pathogens is important to avoid overtreatment of febrile syndromes with antibiotics. Miniaturization of tests through use of micro- and nanotechnologies combines several advantages: miniaturization reduces sample requirements, reduces the use of consumables and reagents leading to a reduction in costs, facilitates parallelization, enables point-of-care use of diagnostic equipment and even reduces the amount of potentially infectious disposables, characteristics that are highly desirable in most healthcare settings. This critical review emphasizes our vision on the importance of multiparametric testing for diagnosing pneumococcal infections in patients with fever and examines recent relevant developments in micro/nanotechnologies to achieve this goal.

Keywords: immunoassays; microfluidics; nanodiagnostics; nanotechnology; pneumococcal disease; point-of-care; rapid tests.

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Introduction

Streptococcus pneumoniae (pneumococcus) is a Gram-positive bacterium that is a major cause of infectious diseases including pneumonia, empyema, sepsis, otitis media and meningitis. Pneumococcal infections occur more frequently in the elderly or the very young. Infection may point to an underlying compromise of the host's immune system but infection can also result from an overwhelming inoculum or a particularly virulent strain. Asymptomatic carriage in the nasopharynx occurs in children below the age of 10 years with a reported prevalence of 30%–60% but is also found in 1%–10% of adults (1). In economically deprived populations and in developing countries, these carriage rates are often higher (2). Here, the detection of the pathogen in the nasopharynx alone, is therefore not sufficient to diagnose a clinical infection. Thus, another parameter is required here to identify the pathogen which emphasizes the need of multiparameter tests.

Streptococcus pneumoniae has several virulent factors (Figure 1 and Table 1) and at least 93 different serotypes are identified (14) the prevalence of which changes over time and differs between geographic locations (15), thus adding some critical potential to the challenge of reliable diagnosis using a single diagnostic assay. Diagnosis is important

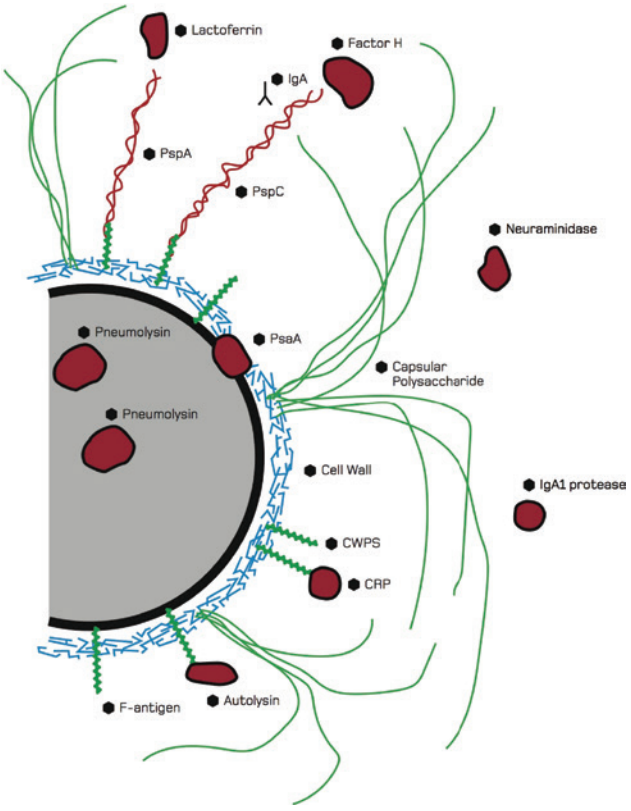


Figure 1: Schematic representation of *S. pneumoniae* and its virulent factors [with permission from textbook in Diagnosis, Serotyping, Virulence Factors and Enzyme-linked Immunosorbent Assay (ELISA) for Measuring Pneumococcal Antibodies." (2nd version) Statens Serum Institut, Denmark (3)].

because untreated disease (e.g. pneumonia, sepsis, meningitis) may be lethal. Specific treatment is available and may be life-saving when applied early. Current diagnostic options are shown in Figure 2. A clinical syndrome compatible with pneumococcal disease is typically worked up with radiographs and antigen testing in developed countries, but in resource poor countries, diagnosis of pneumonia, meningitis and sepsis is often based on clinical algorithms only (16). The resulting overuse of antibiotics is believed to contribute to the development of antibacterial drug resistance.

In this critical review we briefly discuss common diagnostic methods (detection of antigens, nucleic acids and serologic response) and illustrate these with relevant achievements (including recent) that were felt to be important. This will be followed by the timeline of testing in a clinical context. In the final part of this work, we present emerging diagnostics that use micro/nanotechnology such as micro/nanoparticles, microsensors, and microfluidic devices. Here, the focus is also on new techniques that might lead to a new era of diagnostics for pneumococcal infections.

Original articles and reviews published from January 2000 to April 2016 were identified by keyword search in PubMed, ScienceDirect and Scopus using the keywords "pneumococcus", "pneumococcal infection", "pneumococcal antigens", "nanotechnology" and following links in identified articles. Articles cited in the review were selected by the authors based on their perceived relevance for the target audience of this journal.

Microscopy and blood culture

The microscopic appearance and colony morphology is used in most clinical laboratories to identify *S. pneumoniae*. Under the microscope, the Gram-positive bacteria is visible as slightly pointed cocci that are usually found in pairs, but also in short chains or as single bacteria. On blood agar plates, most serotypes of this microorganism form small round doughnut shaped colonies. Serotype 3 and 37 typically form large mucoid colonies (17).

Streptococcus pneumoniae is capable of producing α-hemolysis on blood agar plates, which produces a green color. Other species as *Streptococcus mitis* and *Streptococcus oralis* also have this characteristic and they are often referred as the viridans group (18). Thus additional specific characteristics of *S. pneumoniae* are required to exclude most viridans species such as optochin susceptibility and catalase negativity (18, 19). Bile solubility might be used, however, some exceptions are known (20). Thus, identifying *S. pneumoniae* on phenotype alone is not adequate enough.

Detecting antigens, nucleic acids and serology

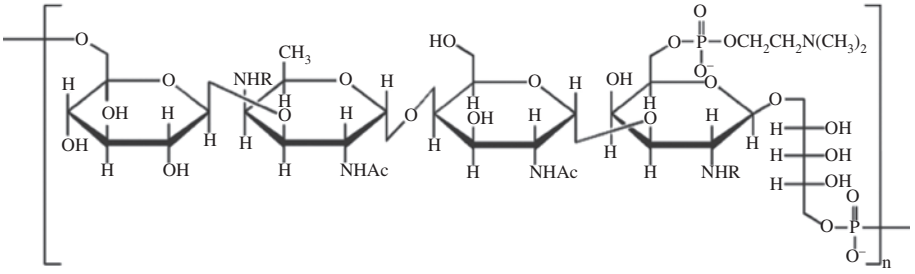
Antigens

Teichoic acids or cell wall polysaccharides

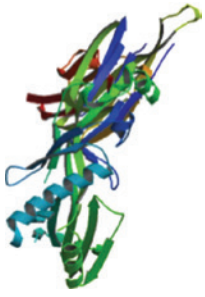
Teichoic acids, found in Gram-positive bacteria, are bacterial polysaccharides of ribitol-, or glycerol-phosphate that are linked via phosphodiester bonds. These anionic glycopolymers, known as cell wall polysaccharide (CWPS), play a key role in antibiotic resistance (21). CWPS can be detected by the commercial Binax NOW (Alere, Global) rapid urinary antigen test, with a clinical test performance reported in a meta-analysis (22). The majority of included patients were adults and had suspected community-acquired pneumonia (CAP); data comparing the Binax NOW test against any reference test were analyzed. In

Table 1: Top, a description of important virulent factors of *S. pneumoniae*.

Virulent factor	Molecular weight	Description	Type
Capsular polysaccharide (CPS)	–	Outermost layer of *S. pneumonia* ±200–400 nm thick; prevents phagocytosis; 93 serotypes (4)	Polysaccharide
Theichoic acids: also called cell wall polysaccharide (CWPS)	–	Common cell wall polysaccharide; tetrasaccharide units, joined together through ribitol phosphate diester and contains one or two phosphocholine subsituents (4)	Polysaccharide
Lipoteichoic acid	–	Also called F-antigen; polysaccharide part (same as CWPS) linked to diacylated glycerol via glucose residue (4)	Polysaccharide
Pneumolysin	53 kDa	Pore-forming protein capable of causing lysis and activation of complement (5)	Protein
Pneumococcal surface protein A (PspA)	67–99 kDa	Bound to cell surface through CWPS/choline attached; inhibits opsonisation and phagocytosis through binding complement component C3 (6, 7)	Protein
Pneumococcal surface protein C (PspC)	75 kDa	Bound to cell surface through CWPS/choline attached protein; mediates adherence to host by recognizing sialic acid on epithelial cells (8, 9)	Protein
Pneumococcal surface adhesin A (PsaA)	37 kDa	Membrane bound; ABC-type transport protein complex; transports Mn2+, plays role in attachment to host cell and virulence (10)	Lipoprotein
N-acetylmuramoyl-L-alanine amidase (Autolysin)	36 kDa	In cell envelope, degrades peptidoglycan in cell wall leading to lysis and release of pneumolysin. Is activated by bile (11)	Enzyme
Neuraminidase A and B [NanA and NanB)	108 kDa (NanA) 75 kDa (NanB)	Sialidase that cleaves the terminal sialic acids from glycoproteins, glycolipids and oligosaccharides on the cell surface and in the mucus (12)	Enzyme
IgAI protease	–	Capable of cleaving human immunoglobulin A1 (IgA1) (13)	Enzyme



CWPS. Tetrasaccharide units. R=H or $COCH_3$



Protein structure pneumolysin

On the bottom (right), the structure formula of the tetrasaccharide units of CWPS and (left) a 3D protein structure of pneumolysin [image with permission from the RCSB PDB (www.rcsb.org) of PDB ID 2bk1. [Tilley SJ, Orlova EV, Gilbert RJ, Andrew PW, Saibil HR. "Structural basis of pore formation by the bacterial toxin pneumolysin." Cell 2015;121:247–56].

children, a high rate of false positives due to the carrier status of pneumococci was found, rendering the test less useful in this setting. In 12 out of the 27 studies, the reference standard was a combination of blood culture, sputum (culture or smear) and culture of another respiratory sample (e.g. nasopharyngeal, transthoracic needle aspirate). Bivariate meta-analysis resulted in an overall sensitivity of 68.5% and a specificity of 84.2% for the Binax NOW test. The inclusion of all 27 selected studies with positive blood culture alone as reference test yielded a sensitivity of 74.0% and a specificity of 97.2%. Other investigators reported that false negative results may arise with low CWPS levels while false positive results may result due to cross-reaction, for example shortly after an infection, in nasopharyngeal carriage or in the presence of *S. mitis* and/or *S. oralis* species carrying closely related CWPS (23).

Capsular polysaccharides (CPS)

Capsular polysaccharides (CPS) form the outermost layer of the pneumococcal wall and their variability results in 93 known serotypes. CPS are detectable in the urine of patients and asymptomatic carriers during, after infection and even after vaccination. Antibodies against CPS are protective rendering CPS suitable as vaccines.

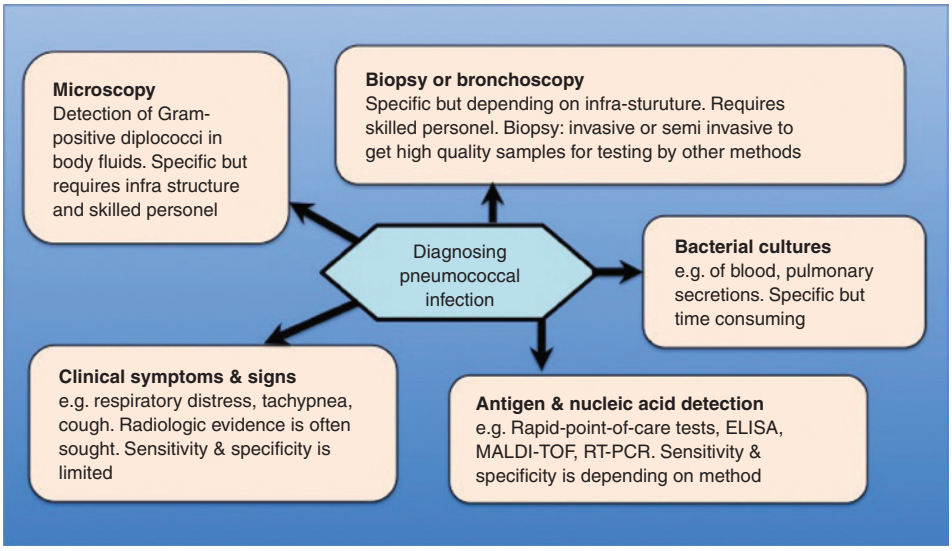


Figure 2: A schematic overview of the different possibilities to diagnose pneumococcal disease.

A multiplexed immunoassay was developed, employing microspheres and based on the Luminex system, which permits detection of 13 different serotypes in urine with a limit of detection of 0.6–8.8 pg/mL (24). A clinical sensitivity and specificity of 97% and 100% was achieved when testing urine samples in the subset of patients with a positive blood culture for *S. pneumoniae* and an X-ray confirmed CAP. Pickering and Hill (25) present a protocol to measure the antibodies after vaccination with the 23 valent pneumococcal polysaccharide vaccine based on the Luminex xMAP (Luminex Corporation, Austin, TX, USA) microsphere-based liquid assay. Sensitivities and specificities are not included.

Serotyping pneumococci by CPS detection in urine or other fluids such as nasopharyngeal secretions is also possible with the latex agglutination test. A protocol for agglutination reagents with serotype specific antibodies from antisera on polystyrene latex particles is available (26). This low-cost protocol is easy to use, includes quality control and allows storage for a year at 2–8°C, and is therefore suited also for resource-constrained settings.

Pneumolysin

The intracellular pore forming toxin pneumolysin is a 53 kDa conserved protein released upon LytA (autolysin) lysis that contributes to the invasiveness of the strain. Pneumolysin detection in sputum, urine, cerebrospinal fluid and blood indicates pneumococcal infection (27).

The value of pneumolysin detection in urine was investigated and compared to the Binax NOW test in adults and children in another investigation (28). The pneumolysin ELISA required 3 h processing time. One hundred and eight patients with blood culture confirmed pneumococcal infections were tested. In adults, sensitivity and specificity were 56.6% and 92.2%, respectively. Pneumolysin concentration in urine decreased after the initiation of treatment. Notably, pneumolysin was not detected in children with only nasopharyngeal colonization, leading to enhanced specificity. In colonized children, sensitivity and specificity of pneumolysin was 62.5% and 94.4%, respectively, while Binax NOW had a sensitivity and specificity of 87.5% and 27.8%, respectively. For non-colonized children, the sensitivity and specificity of the pneumolysin ELISA was 68.7% and 94.1%, respectively, while the Binax NOW test had a sensitivity of 93.7% and a specificity of 41.2% (28).

In another investigation, the Binax NOW test was used to detect pneumolysin in cerebrospinal fluid (CSF) and compared to culture and latex agglutination (29). 1173 CSF samples of five countries in Africa and Asia, collected from patients between 1 and 59 months old. From these patients, n=69, were confirmed by culture on pneumococcal meningitis and the Binax NOW test was found to be positive in 98.6%. By using the test on culture positive samples for bacterial meningitis caused by other pathogens (n=125), the test was negative in 99.3%. When only the latex agglutination tests and culture was used, pneumococci were detected from 7.4% in Asia to 15.6% in Africa, including the Binax Now test resulted in 16.2% in Africa (Nigeria) to 20% in Asia (Bangladesh).

These results suggested the underestimation of pneumococci infections in the past.

Other pneumococcal antigens

Pneumococcal surface adhesin A is an immunogenic, 37 kDa surface lipoprotein expressed by all pneumococci. It is highly conserved among pneumococcal serotypes, and is also present in *S. anginosus, S. mitis* and *S. oralis* with a sequence similarity of 90%, 94% and 95% compared to *S. pneumoniae* serotype 6B (30), limiting its diagnostic value.

Others investigated surface proteins are the pneumococcal surface protein A (PspA), family 1 and 2, and the pneumococcal surface protein C (PspC). PspC, present in 75% of pneumococcal strains, is related to PspA, but has a specific N-terminal region interfering with the complement system through binding of the complement factor H. The Hic protein, (factor H-binding inhibitor of complement), is a PspC variant mainly found in serotype 3 (31). The homology between these proteins renders their specific discrimination difficult and may limit their value as a diagnostic biomarker.

The triggering receptor expressed on myeloid cells (sTREM1), has been reported as a promising marker for infection (32), and has a decent correlation with blood culture positivity in a univariate meta-regression analysis of 13 studies. The pooled sensitivity and specificity was 84% and 77%, respectively, for the diagnosis of lower respiratory tract infection with similar diagnostic accuracy for community-acquired infection and hospital acquired infection in subgroup analysis.

The search for new potential biomarkers by using mass spectroscopy-based proteomic analysis in African children provided two valuable candidates: lipocalin-2 and haptoglobin (33). Lipocalin-2 discriminated non-severe and non-bacterial from severe and bacterial pneumonia and the authors hypothesize that the use of this marker, embedded in a point-of-care-test in combination with haptoglobin measurement, could distinguish severe pneumonia from malaria and viral lung infection.

Nucleic acids

Pneumococcal nucleic acids can be detected by amplification technologies. The polymerase chain reaction (PCR) amplifies a selected region of the target gene, but is challenged by the homology of genes between species and the occurrence of false positive results in asymptomatic carriers. Strain-specific primers focusing on non-homologous DNA regions may improve the specificity of amplification tests. An example of pitfalls of homology among bacterial species, is the pneumolysin gene *ply* as reported by Song et al (17). Samples originating from the lower respiratory tract of patients with pneumococcal disease were tested and yielded sensitivities between 68% and 100% and poor specificities. However, throat swabs of patients with community acquired pneumonia and from control subjects resulted in similar rates of test positivity of ~55%–58% for the *ply* gene (34). Another example is the *lytA* gene that is present in pneumococci as well as in streptococci, but varies less among streptococci. Others found that in ~2% of the investigated pneumococcal strains a mutation is present that is resulting in a negative PCR test (20). This test is still applicable in routine laboratories but needs confirmation in case of a negative result. To distinguish between α-hemolytic streptococci and pneumococci the bile test might be used. The cell wall of pneumococci is bile soluble (and therefore resulting in lysis), where as all α-hemolytic streptococci are not. However, the 2% pneumococci carrying the mutation in the *lytA* gene are also bile insoluble and thus providing a false negative result.

Thus, strain specific primers may be of high importance for certain microorganisms and might improve the sensitivity and specificity of an amplification test. A high-throughput method for serotype specific quantification and molecular serotyping of pneumococci using a nanofluidics based RT-PCR system was developed (35). Primer pairs were designed such that over 50 serotypes are covered. Their performance is comparable to the conventional PCR-based assays. Recently, a novel quantitative real-time PCR was developed to detect 40 major pneumococci serotypes worldwide directly in blood and nasopharyngeal specimens (36). This work presents newly designed primers and probes based on targets that were already described by Pai et al. (37). The most frequent serotypes found in Brazilian, French and South African (see Figure 3) samples were 14, 1 and 7A/F and 3 and 19F, respectively. In case both blood and nasopharynx samples were available, the serotype in blood was always present together with other serotypes in the nasopharynx. This highly sensitive assay was capable of detecting <100 CFU/mL and opens the door to large-scale epidemiological studies of pneumococci. Other scientists (38) published a novel quantitative PCR assay for the detection of *S. pneumoniae*. The target is the competence regulator gene *comX*, which exists in duplicate copies in *S. pneumoniae* and not in other bacterial species. Validation of the assay was done on DNA extracted from serum of 30 patients with blood cultures positive for *S. pneumoniae*

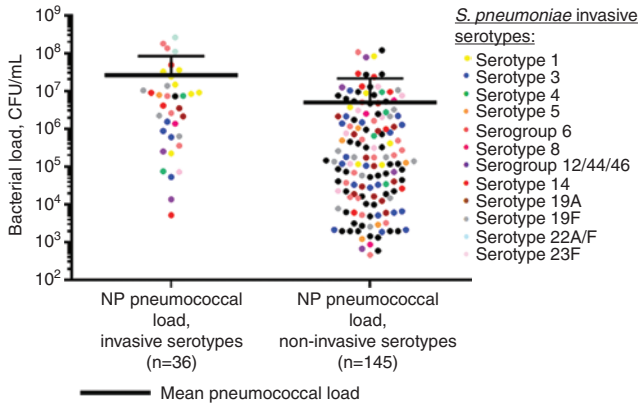


Figure 3: Pneumococcal load distribution in nasopharyngeal samples from the South African cohort.
Each plot represents a sample and the mean bacterial concentration for each group is indicated by the bold bars (Student's t-test, $p<0.001$). Invasive and non-invasive serotypes are compared upon presence in nasopharyngeal samples en whole blood. [Messaoudi et al. (36); http://dx.doi.org/10.1371/journal.pone.0151428.g002.]

and 51 serum samples positive for other bacteria. The *LytA* quantitative PCR assay was used to compare. The clinical sensitivity was 47% for both assays and the diagnostic specificity was 98.2% and 100% for *LytA* and *comX*, respectively (38).

Serology

The detection of serotype specific antibodies (CPS) can be done by ELISA, the Neufeld Quellungs test and the latex agglutination test [see also *Streptococcus pneumoniae* Textbook, Statens Serum Institut, Copenhagen, Denmark (3)].

Immune response against, for example, PspA, PspC and Hic are known, but titers do not sufficiently discriminate patients from healthy controls, with the exception of PspA that shows a significant titer increase in the convalescence phase. Cross-reactivity between the antibodies for PspA and PspC are also reported (39, 40). Multiparameter testing is probably required for correct result interpretation in this situation.

A validation study for a multiplexed set of antigens causing respiratory tract disease was performed where fluorescent bead based multiplexed immunoassay quantifies IgG against a panel of microorganisms including *S. pneumoniae*, *Haemophilus influenzae* and *Moraxella catarrhalis*. For each marker a singleplex measurement was compared with the multiplex assay, and the pneumococci multiplex was compared with an ELISA of 22 pneumococcal antibodies containing serum samples that had been analyzed previously. The reproducibility, specificity and correlation were validated for six pneumococcal antigens [Ply, PspA1, PspA2, choline binding protein A (CbpA), pneumococcal choline binding protein A (PcsA) and pneumococcal histidine triad protein D (PhtD)]. The assay had a specificity above 92% and some cross-reactivity between PspA1, PspA2 and CbpA was reported. The serum samples of 50 children were also examined and revealed a wide range of antibody concentrations and increases in samples of recovering patients (41).

Time line of testing

Applying a test in the right time slot of infection is critical for its clinical benefit and its interpretation. Blood culture testing is more sensitive early in infection, but becomes rapidly negative after treatment with antibiotic therapy. Recently, a strategy was proposed to identify various bacteria including pneumococci within 6 h after signaling growth in blood culture (42). Directly from the positive blood cultures, rapid agglutination tests were performed and showed a very high predictive value. Such early diagnosis allows faster treatment adjustment in serious bacterial infection. Negative tests were repeated after colonies grew for further 4–6 h.

DNA based detection of pneumococci in serum was found to be particularly sensitive when the disease was more severe than uncomplicated pneumonia, and claimed that such testing gives additional diagnostic benefits to blood cultures including: 1) better disease severity assessment, 2) antibiotic streamlining and 3) detection of invasive pneumococcal disease after initiation of antibiotics (43).

Others investigated the use of a urinary test after starting an empiric antibiotic treatment and concluded that this has a potential to guide the right choice of medical treatment of pneumococcal disease in adults at an earlier stage (44). From this we conclude that combining existing and new test modalities may lead to new improved diagnostic strategies/management.

Towards multiparameter testing

Multiparameter testing includes the notion of determining multiple parameters for a particular infectious agent, and also the concept of testing for multiple pathogens in a single test. An important technique here, which evolved

rapidly last decade, is the use of matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry (MS) in the clinic. This technique enables the rapid detection of bacteria and fungi in <1 h when starting with pure bacteria culture. The advantage is that there is no knowledge of the pathogen required in advance, however, the disadvantage is that a pure culture is required and with this sufficient equipped laboratory and trained personnel. Thus, for most resource poor countries this is not an option. Fall et al. (45), presented the use of MALDI-TOF in Dakar (Senegal) where it was successfully used to identify species causing infectious diseases in tropical Africa.

While testing in febrile disease in developed countries often yields an abundance of parameters from a fully equipped central lab as mentioned above, multiparameter testing in a simple and inexpensive fashion is a pressing need in particular for these countries. Mortality and morbidity resulting from infectious diseases are still highest in sub-Saharan Africa and in low-income countries of southeast Asia. The relative frequency of fever episodes due to pneumococcal disease, malaria, typhoid fever, dengue, or chikungunya may change with age, with season, as a function of geography, and may be related to local disease outbreaks. Currently, blind treatment is associated with high costs and microbial resistance development, while systematic testing is economically unrealistic in many areas. The recent emergence of global viral threats including new influenza subtypes, dengue virus, Middle East respiratory syndrome coronavirus and Ebola add an additional urgency to the development of rapid, bedside, low-cost tests that discriminate between multiple alternative causes of fever.

There is evidence that availability of multiple diagnostic parameters may enhance the diagnosis of pneumococal disease. The combination of a positive urinary Binax Now antigen test combined with measurement of CRP and PCT in plasma was evaluated in children (<6 years) (46). Diagnostic accuracy for predicting pneumococcal CAP (without positive urine test) for PCT alone (cut-off≥1.5 ng/mL) was limited, having a sensitivity of 94.4% and a specificity of 52.6% whereas for CRP (cut-off≥100 mg/L) the sensitivity was 91.9% and the specificity 60.5%. In combination with a positive urine test (same cut-offs), sensitivity and specificity were 65.5% and 85.7% for PCT, and 65.5% and 88.6% for CRP, respectively. Thus, in case of an emergency consultation such a combination is a useful tool to predict presumed pneumococcal CAP. Applied to examine pleural fluid samples of children, the Binax NOW had a sensitivity of 71%–96% and a specificity of 71%–100% for pneumococcal empyema (47–50). Recent publications show progress in miniaturization of diagnostic technologies, paving the way to multiparameter testing by integration of multiple assays into a single device or test substrate [for further reading see (51, 52)].

Micro/nanotechnology diagnostic innovations

Both micro- and nanotechnology has significantly contributed to new therapies, drugs and diagnostics during the last 5–10 years. In this paragraph, we only focus on new developments that might be used to improve or to develop new diagnostics for the detection of a pneumococcal infection.

The separation of bacteria from blood (or urine) may be done by, e.g. filtration, centrifugation and sedimentation. However, the key limitation for *S. pneumoniae* is that the number in colony forming units (i.e. viable bacteria) in blood cultures is known to be low and high sensitivity is improbable except when a large volume of blood is separated. The use of microfluidic devices may largely improve the capability to separate or concentrate bacteria prior the use of other diagnostic methods and tests. For example, the work of Park et al. (53), who developed a microfluidic device for the continuous dielectrophoretic separation and concentration of bacteria from crude biologic samples. They showed a 104 concentration of target cells and provided a separation efficiency of 94.3% in human CSF and 87.2% in blood for *Escherichia coli*. Ai et al. (54) used a microfluidic device and surface acoustic waves across the channels and demonstrated the separation of *E. coli* bacteria from peripheral blood. Another possibility is size-based cell sorting by the use of an ordered array of obstacles. This is also called deterministic lateral displacement. A combination of grooves and protrusions in a microfluidic device could successfully separate, 3D spherical particles, red blood cells (2D planar shaped) and rod shaped bacteria. The bacteria investigated in this work were: *E. coli* (rod-shaped or bacillus), *K. pneumoniae* (rod shaped), *S. epidermidis* (spherical or coccus) and *Pseudomonas aeruginosa* (rod-like or coccobacillus) (55). The final example is the work of Kang et al. (56) who presented a method to selectively detect bacteria directly from milliliters of diluted blood in one step. Droplet microencapsulation of diluted blood was done by using a microfluidic device and a high throughput 3D particle counter system. Specific DNA enzymes were used as sensor to the selected target *E. coli*. The fluorophore attached the DNA enzyme will be released from its quencher upon binding

and subsequently generate a fluorescent signal. The limit of detection was 1–10 CFU/mL. To conclude, the use of microfluidic techniques to separate and/or concentrate and detect bacteria is promising but needs to be further developed for challenging bacteria as *S. pneumococci*. In addition, the above mentioned is mainly applicable in developed countries.

Micro- and nanoparticles are nowadays widely used in lateral flow tests (rapid tests) that are in most cases based on sandwich immunoassays. Here, the presence of an antigen or antibody in a liquid sample (e.g. blood, serum, plasma, urine and CSF) leads to the immobilization of micro/nanoparticles and a visible signal. Examples of used particles are gold nanoparticles, colored latex beads, magnetic particles, carbon nanoparticles. Important here is that all materials should retain their properties once conjugated to biomolecules and should be easily detectable at a concentration near the diagnostic threshold. [For further reading, see Refs. (57–59).] Lateral flow tests for the detection of pneumococcal already exist, however, an option would be to develop multiparameter tests as mentioned in the previous paragraph (46).

Wu and coworkers (60) presented a novel detection system based on quantum dots and microbeads that was developed to detect target DNA of pathogenic bacteria. On microbeads DNA hairpin structures are coupled. The composition of these hairpin oligonucleotides includes: a poly-T linker, a Tag sequence, the barcode region and an anti-Tag sequence. The barcode region is composed of four words each of four nucleotides (61) and has a unique design used for each species. Then, two types of probes are used: the internal probe, which has a complementary sequence to the barcode region and a reporter probe that has a sequence complementary to the anti-Tag region. Both probes have quantum dots in two different emission spectra. *Legionella* spp. is used as an example microorganism. The method exists of four steps, 1) the DNA hairpin structures on the microbeads will be denatured at 95°C, then 2) the provided linear oligonucleotide microbeads will hybridize with the denatured DNA samples (*Legionella* spp.) and will prevent the recovery of the hairpin structures, 3) the linear oligonucleotides will now be able to hybridize with both the internal as the reporter probes. When the target DNA is not present, the hairpin structure will recover. The fluorescence intensity of the reporter probes may be used to quantify the target DNA. The limit of detection was 0.1 ng of the extracted DNA and 10 CFU/test. This method is very useful for the multiplex detection of serotypes of *S. pneumoniae,* however, will only be available for well-equipped laboratories.

Veigas et al. (62) showed for the first time that by functionalizing a single Au-nanoprobe with multiple sequences, in the presence or absence of two pathogens could be determined in a single test. The selected targets were the conserved region of the *Mycobacterium tuberculosis rpoB* gene and *Plasmodium* 18S ribosomal RNA (18S rRNA). After a multiplex PCR reaction, a colorimetric assay is performed by heating each sample up to 95°C (concentration 60 μg/mL) and cooled down to 25°C in the presence of the functionalized Au-probes. The assay consist of a blank (sample without DNA), non-related control DNA and the samples. $MgCl_2$ in a pre-determined concentration was added to the samples after 30 min for color development. After measurement by UV/Vis spectroscopy, the aggregation profiles for each Au-nanoprobe were analyzed by comparing the absorption ratio 525 nm/600 nm. This method showed the capability of these particles. As also suggested by the authors, replacing the PCR by loop mediated isothermal amplification (LAMP) and the use of a cheap disposable platform (paper) as was already presented in another investigation of the authors (63) will simplify the use of this method in resource poor areas.

LAMP assays are already developed and integrated on microfluidic platforms as described by Luo et al. (64). They showed that the differentiation of bacterial strains in respiratory tract infections could also be done in multiplexed nucleic acid detection assays. The developed microfluidic device for LAMP was successfully used for the detection of *H. influenza*, *K. pneumonia* and *M. tuberculosis* with limits of detection of 17, 16 and 28 copies per μL, respectively. Methylene blue was used to electrochemically indicate the presence of double stranded DNA (65). This operationally simple and potentially fast and cost-effective device analysed multiple genes qualitatively and quantitatively paves the way to multiplexed assays including other organisms as *S. pneumoniae*.

From particles, we move to silver nanorods and surface enhanced Raman scattering (SERS). Kotanen et al. (66) characterized and evaluated a handheld SERS system and showed that it can identify bacteria in pooled human sera. Different bacteria (*Acinetobacter baumannii, P. aeruginosa, K. pneumoniar, E. coli and Staphylococcus aureus*) were individually inoculated into pooled human sera. Lysis filtration was performed to separate and isolate the bacteria. Silver nanorod substrates were incubated with the bacterial samples for 3 h at 60°C. The molecular fingerprint of each species was determined by using partial least squared differential and principal component analysis. With the device, they could were able identify bacteria at the species level from both serum as culture samples with a limit of detection of 10^9 CFU/mL. Identification at strain

level was not obtained. Therefore, it was suggested that the spectrometer instrumentation needs to be at an equal level with the measurement sensitivity of the used nanoparticles which are known to be capable of sufficient enhancement factors to discriminate in between strains (67).

Even when we estimate that the actual production and consumable costs are too high for developing countries, we do see a potential for this portable device for future use in resource poor areas. Battery based hand-held spectrometers with capacities to transfer the provided bacterial fingerprints by satellite based Internet to a central laboratory is not unrealistic. For further reading about isolation and identification of bacteria by means of Raman spectroscopy, see Ref. (68).

On-chip microbial growth was combined with sensitive and specific surface plasmon resonance detection (SPR) of target antigen binding to an immobilized detection antibody (69). The fluidic-less device has the capacity to culture-capture and measure bacteria as a result of coupling a microarray to a surface resonance imager. This enables the label free and real-time monitoring of bacteria with a series of immobilized ligands. Different types of viable cells growing and dividing on chip were successfully detected by SPR imaging with a limit of 2.8±19.6 CFU/mL in the initial sample. On-chip, *S. enterica, S. pneumoniae* and *E. coli* O157:H7 was assayed and quantitative determination of the initial contaminating bacteria was possible (validated with standard plate counting data). The method developed proved to produce within only a few hours (with liquid or solid samples), similar results to culturing methods (sensitivity and specificity) that were run in parallel and over several days. The developed biosensor has the advantage of being simply to use, needs minimal supplementary handling and is promising for the future use in resource low areas. More details about SPR can be found in Ref. (70).

A nanomechanical olfactory sensor system for the detection of volatiles was developed and tested (see Figure 4) (71, 72). This device consists of an array of silicon cantilevers with differential surface functionalization by polymers that swell upon exposure to various vapor mixtures (containing volatile molecules such as alcanes, alcohols, aldehydes). The complex nanomechanical response pattern was fed into an artificial neuronal network for pattern recognition. The diagnosis of respiratory failure was shown with a sensitivity of 83%, a specificity of 95% and a diagnostic accuracy of 89%. This work is followed by others who developed an electronic nose-on-chip to detect metabolites generated during infection capable for the diagnosis of ventilator-associated pneumonia (73). Using a kernel learning method, a classification accuracy for the infected samples of 100% was reported. This device might be used for rapid diagnosis and might even be usable in resource poor areas.

Multiparameter testing may require expertise for correct interpretation, which may depend on specific clinical settings, on specific markers combinations and potentially on differential parameter thresholds. Advanced analysis strategies implemented in software and accompanied by suited user interfaces will therefore be an important complement to the actual biomarker tests.

Conclusion

The diagnosis and management of pneumococcal disease remains challenging, in particular in children who are

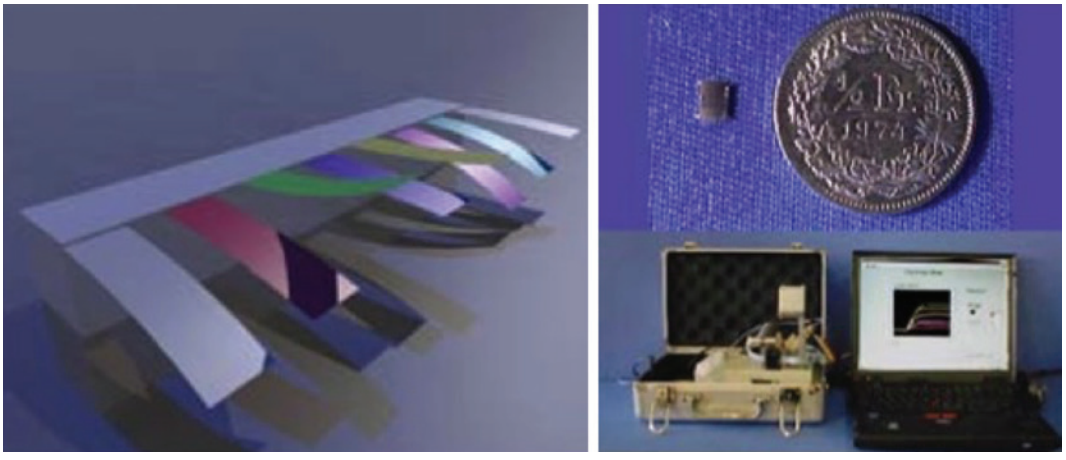


Figure 4: Left, a differentially polymer coated cantilever array.
Any changes in the surface tension lead to bending of the cantilever and can be measured by the laser that is integrated in the system. Right, (top) the chip with the sensor array, (bottom) the entire analysis system [with permission from Ref. (71)].

often asymptomatic carriers, and in low-income countries with a high morbidity and mortality from febrile illnesses caused by a range of bacteria, viruses and parasites.

New biomarkers were recently identified that add information about the presence as well as the invasiveness markers of pneumococci. Nucleic acid detection technology for bacteria progresses rapidly, although the risk of false positives due to asymptomatic bacterial colonization exists.

Combining multiple parameters, e.g. adding inflammation marker quantification to urine CWPS antigen detection has proven valuable in current clinical practice and in clinical studies to determine true positives, but there is room for improvement. Detection of markers of invasiveness (e.g. pneumolysin detection) and of specific nucleic acid sequences is desirable to optimize sensitivity and specificity in parallel, and to gather additional information about the threat level of the detected pathogen. Integration of multiple markers into a single, rapid test could add additional value to the diagnostic approach. Likewise, development of multiparameter tests for relevant arrays of pathogens is important to avoid overtreatment of febrile syndromes with antibiotics, and such test sets might be optimized for individual geographic or epidemiologic settings. In the best of all worlds and in view of the scarce resources in the developing countries and increasingly also in the healthcare systems of developed countries, such tests would be inexpensive, too, to benefit as many patients as possible.

A caveat is the antigenic and genetic drift of pathogenic microorganisms, which may require tuning of available tests to changing epidemiologic settings, triggered by continuous monitoring of circulating microorganisms similar to what is currently done in influenza. Miniaturization of tests through the use of micro- and nanotechnologies is a clearly observable trend because of several advantages: miniaturization reduces sample requirements, minimizes the use of consumables, facilitates parallelisation, enables point-of-care use of diagnostic equipment and even reduces the amount of potentially infectious disposables, characteristics that are highly desirable in most healthcare settings.

Acknowledgments: We thank Prof. Rutledge Ellis-Behnke of the Medical Faculty Mannheim of the University of Heidelberg (Germany) for the support in knowledge. This review has been written to contribute to the DiscoGnosis project that has the core objective to develop a platform that would allow the detection of malaria and similar pathogenic diseases in a rapid, multiplexed and non-invasive. The project is supported by the European Commission through the 7th Framework Programme on Research and Technological Development within the Objective FP7 ICT-2011.3.2 and under Grant Agreement [No. 318408].

Conflict of interest statement: Authors state no conflict of interest. All authors have read the Journal's publication ethics and publication malpractice statement available at the journal's website and hereby confirm that they comply with all its parts applicable to the present scientific work.

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Bionotes

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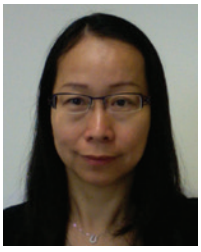

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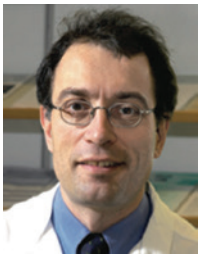

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