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REVIEW

Laboratory characteristics of *streptococcus pneumoniae*: A persistent pathogen

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Abstract
Despite advances, such as the availability of an effective vaccine against Streptococcus pneumoniae, the organism still contributes significantly to morbidity and mortality worldwide, especially in children. It is crucial that accurate identification and susceptibility testing of the organism occurs, to allow for accurate estimation of disease burden, development of vaccines, and initiation of optimal treatment. Herein the laboratory diagnosis, susceptibility testing and treatment of S. pneumoniae is discussed.

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Background

Despite the introduction of vaccines against *Streptococcus pneumoniae*, it still contributes significantly to morbidity and mortality worldwide.[1] It causes invasive disease including meningitis, bacteraemia, or infection of other sterile sites such as empyema and pericarditis.[2,3] Less severe, but still important non-invasive manifestations include pneumonia in the absence of bacteraemia, otitis media and sinusitis.[3]

It is estimated that in the year 2000, pneumococcal infections were responsible for 600 000 deaths in HIV-uninfected children, and an additional 95 200 deaths in HIV-infected children.[1] After 2010, many countries with a high burden of pneumococcal disease and who were eligible for vaccine funding from Gavi (the Global Vaccine Initiative Alliance), introduced the pneumococcal conjugate vaccine (PCV) as part of their national immunization programme.[1] Between 2000 and 2015, deaths due to pneumococcal infection declined by 51% in HIV-uninfected children, and 71% in HIV-infected children.[1]

However, despite this large-reported reduction in the proportion of disease from 2000-2015, in 2015, 294 000 HIV-uninfected and 23 300 HIV-infected children are still estimated to have died, highlighting the continued role pneumococcal disease still plays in childhood mortality.[1] India, Nigeria, The Democratic Republic of Congo, and Pakistan accounted for approximately 50% of these deaths in 2015, and sustained efforts to increase PCV vaccine coverage in these countries and others that have not yet introduced the vaccine, is necessary to combat the burden of disease particularly in children of low socio-economic status who are at increased risk of death.[1]

Misidentification of S. pneumoniae not only affects the estimation of disease burden, it also affects the development of vaccines against prevalent strains, and can also delay the initiation of optimal treatment. This is a frequently encountered problem in developing countries that still largely rely on culture-based methods for the identification of this organism. This article summarises the laboratory characteristics of S. pneumoniae and briefly expands on susceptibility testing and its implications in the treatment of this important pathogen, thereby providing a summary for clinicians and laboratory personnel alike.

Laboratory Diagnosis

Microscopy and culture are used commonly in diagnostic labs for identification. Gram stain typically reveals lancet-shaped, gram-positive diplococci (pairs) or cocci in short chains, Figure 1A. Most isolates contain an extracellular polysaccharide capsule which can also be visualized on Gram stain (Figure 1A) and by the Quellung reaction. The Quellung reaction is the gold standard for serotyping *S. pneumoniae*. The technique involves exposing the pneumococcus to anticapsular antisera resulting in visual enhancement of its capsule which appears as a halo surrounding the bacteria when viewed under a microscope.[3,4,5]

S. pneumoniae can be cultured on blood and chocolate agar plates that are incubated for 24-48 hours at 35-37° C. Growth is enhanced in 5% CO_2. Colonies appear small and grey with a surrounding green zone, caused by partial destruction of red blood cells, that is given the name α-haemolysis, Figure 1B.[3,4] These colonies often develop a central depression resulting in a draughtsman appearance, Figure 1B.[3,4] Some serotypes, for example serotype three, produce very mucoid colonies. *S. pneumoniae* is differentiated phenotypically from other α-haemolytic streptococci like viridans streptococci by optochin susceptibility.[3,4] Optochin (ethylhydrocupreine) is an antibacterial agent only used for laboratory identification of *S. pneumoniae.*[3,4] Unlike viridans streptococci, *S. pneumoniae* typically display optochin susceptibility (zone size ≥ 14 mm) (Figure 1C) although occasional optochin-resistant isolates have been reported.[3,4,6] Additionally, bile solubility testing, can be used when results of optochin testing is equivocal, and is based on accelerated autolysis of *S. pneumoniae* in the presence of sodium deoxycholate.[3,4]

Matrix-assisted laser desorption ionization time-of flight (MALDI-TOF MS) mass spectrometry is an alternative automated method for identification of *S. pneumoniae*. However, this method is limited in its ability to accurately distinguish between *S. pneumoniae* and the *S. mitis* group due to their genetic similarities. Distinction between these species has been improved by the combined use of a novel algorithm with an expanded MALDI Biotyper database.[7] Some laboratories will still confirm these results using optochin susceptibility. [8]

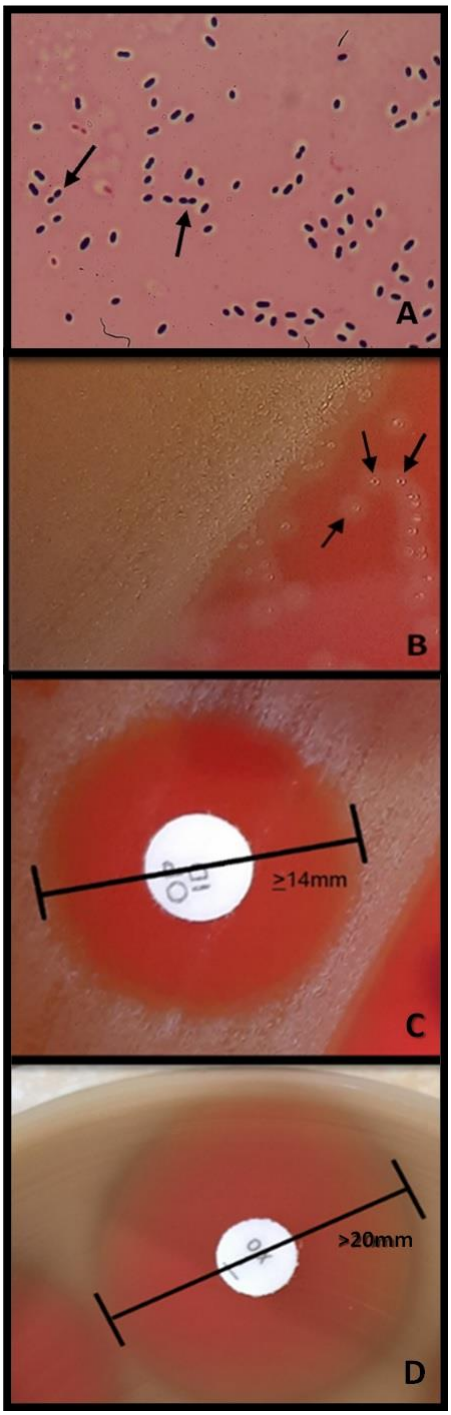

Figure 1. Routine tests performed in the diagnostic laboratory for identification and susceptibility of S. Pneumoniae **(A)** Gram stain showing lancet shaped gram-positive diplococci (purple) with surrounding capsule (arrow). **(B)** S. pneumoniae colonies with draughtsman appearance and surrounding green zone of α-haemolysis on a blood agar plate. **(C**) Optochin susceptibility testing indicating that the isolate is S. pneumoniae due to a zone of inhibition ≥14 mm around the optochin disc. **(D)** Oxacillin disc diffusion screening test with an oxacillin inhibition zone >20mm indicating absence of β-lactam resistance mechanisms.

Serotyping is an important tool for the surveillance of *S. pneumoniae*. This is necessary for monitoring the spread of disease and development of vaccines against the most prevalent disease-causing

serotypes.[9] Geographical distribution, age of affected population and virulence is dependent on the specific serotype identified.[9] Ninety-eight different pneumococcal serotypes have been identified. based on the composition of the polysaccharide capsule.[10] Various methods for serotyping exist, these include capsular typing using antisera (Quellung reaction), MALDI-TOF MS and molecular techniques based on sequencing of the capsule gene.[10] Molecular methods include PCR, immunoblot, microarray and whole genome sequencing.[9,10]

Limitations of culture include the autolysing and fastidious nature of the organism. Additional factors such as low bacterial load of the organism within the sample, delays in transport, inoculation, and incubation of samples as well as the initiation of antibiotic therapy prior to sample collection can lead to false negative culture results. To counteract these limitations, non-culture-based diagnostics have been developed and include:

1) Immunochromatographic antigen detection tests (ICT). These are lateral flow assays and have a rapid turnaround time. They include the BinaxNOW assay which is a urinary antigen test that detects the C-polysaccharide cell wall antigen common to all *S. pneumoniae* serotypes.[4] They have also been used in non-urine samples such as cerebrospinal fluid and pleural fluid.[4] Their role is mainly as an adjunctive tool for diagnosis. Reports of cross-reactivity with other viridans streptococci have been described.[4] The value of the tests in diagnosing infections in children is questionable as nasopharyngeal carriage of *S. pneumoniae* can give rise to positive results.[4]

2) Molecular based methods including PCR. The common pneumococcal gene targets include *ply*, *lyt*A, *psa*A, and *pia*B. In culture negative cases a *lytA* PCR can be supplemented with *piaB* for better identification. However, due to genetic similarity between *S. pneumoniae* and viridans streptococci, cross-reactivity has been described.[9] For this reason, other molecular methods including 16S rRNA sequencing, PCR followed by restriction fragment length polymorphism analysis (RFLP), Multi-Locus Sequence Typing (MLST) and whole genome sequencing are being investigated for accurate identification of *S. pneumoniae* but are beyond the scope of the routine diagnostic laboratory.[9]

Treatment and susceptibility testing

β-Lactam antibiotics such as penicillin, ampicillin, ceftriaxone, and cefotaxime, are the preferred treatment options for meningitis and serious non-meningitis infections. For meningitis, alternative agents include vancomycin. For non-meningitis infections, alternative agents include macrolides, tetracyclines and levofloxacin. The latter two, may be used for treatment in adults, however their use is contraindicated in children.[5]

Prior to 2008, penicillin minimum inhibitory concentration (MIC) breakpoints to determine susceptibility for all *S. pneumoniae* infections, were based on the more conservative values used for pneumococcal meningitis (0.06ug/ml).[11] As such, an increasing number of penicillin non-susceptible (non-wild type) *S. pneumoniae* isolates were reported.[11] These isolates showed reduced susceptibility to penicillin due to altered penicillin binding proteins, which results in lower affinity for β-lactams.[12] This finding led to the increased use of broad-spectrum antibiotics in the treatment of these infections irrespective of the site of infection.[11] However, studies subsequently demonstrated that patients treated with optimal doses of parental penicillin for non-meningitis infections caused by these non-wild type strains with low level resistance (0.12-2ug/ml) had the same outcomes as patients treated with other antibiotics.[11] This led to the revision and differentiation of breakpoints based on the site of infection (meningitis versus non-meningitis), allowing clinicians to safely use parental penicillin to treat non-meningitis pneumococcal infection with low-level penicillin resistance (0.12-2ug/ml) again. For meningitis infections, the breakpoints for parental penicillin have remained low and unchanged at 0.06ug/ml, due to increased mortality when treated with penicillin in the setting of low-level resistance (MIC 0.12-2ug/ml).[11] However, in non-meningitis infections, the breakpoints for parental penicillin were revised to ≤2ug/ml for susceptibility. Similarly different breakpoints for susceptibility to cefotaxime and ceftriaxone exist based on whether the infection is a meningitis or non-meningitis infection.[12,13]

To exclude β-lactam resistance mechanisms, a screening test using disk diffusion with an oxacillin (1ug) disk can be performed. When the screen is negative (oxacillin inhibition zone ≥20 mm), all β-lactam agents for which clinical breakpoints are available, can be reported as susceptible, Figure 1D. When the screen is positive (oxacillin zone <20 mm), MICs to these β-lactams need to be performed to determine susceptibility.[12,13] When the oxacillin zone diameter is <8 mm, this could indicate resistance to broad spectrum antibiotics such as ceftriaxone/cefotaxime.[12] In these cases, an alternative, non β-lactam antibiotic should be added for serious infection until the MICs of the β-lactams are available.[5,12] An example of this would be adding vancomycin to β-lactam antibiotic therapy for pneumococcal meningitis until β-lactam susceptibility is confirmed.

Conclusion

Despite advances such as the availability of an effective vaccine against *S. pneumoniae* serotypes causing infection, the organism remains a formidable foe. It is crucial that accurate identification and susceptibility testing of the organism occurs to allow for accurate estimation of disease burden, development of vaccines against prevalent strains, and initiation of optimal treatment.

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