Journal of Microbiological Methods 141 (2017) 48–54

Contents lists available at ScienceDirect

Journal of Microbiological Methods

journal homepage: www.elsevier.com/locate/jmicmeth

Review

Current challenges in the accurate identification of *Streptococcus pneumoniae* and its serogroups/serotypes in the vaccine era

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ARTICLE INFO

Keywords:
S. pneumoniae
Accurate identification
Molecular methods
Serotyping
Limitations

ABSTRACT

Streptococcus pneumoniae is a major cause of pneumonia, meningitis and other invasive diseases resulting in high mortality and morbidity among children under the age of five. Inaccurate identification of *S. pneumoniae* masks the exact estimation of disease burden and could delay treatment options. This is the common problem most frequently faced in developing countries due to several reasons that include poor infrastructure, insensitive operational procedures and lack of expertise. Inconsistent methods for phenotypic detection often delay the early identification and confirmation of *S. pneumoniae*. For serotyping *S. pneumoniae*, Quellung method is the gold standard which can be performed only on viable isolates, needs expertise and is expensive. Therefore, the data available on disease burden and serotype prevalence is not truly estimated in most of the developing countries, in turn, the use of available pneumococcal vaccines have been restricted. This current review deliberates an overview on advantages and limitations of routinely used phenotypic tests for *S. pneumoniae* identification. Also discussed in this review are the roles and current challenges faced by various molecular identification and serogroup/serotype identification methods of *S. pneumoniae*, including PCR, real time PCR, sequence analysis of different specific genes of *S. pneumoniae*, PCR combined with RFLP, MALDI-TOF, MLST, MLSA and WGS.

1. Introduction

Streptococcus pneumoniae belongs to the mitis group of streptococci that normally (Facklam, 2002) exists as a commensal flora of the upper respiratory tract (URT) (Gray et al., 1980). It is one of the major causes of acute otitis media, pneumonia and in its severe (invasive) form, causes bacteremia and meningitis (MMWR, 2000; Musher, 1992) in children leading to high morbidity and mortality, especially in developing countries (CDC, 2013). Among the mitis group, other closely related species such as *S. mitis*, *S. oralis*, and *S. pseudopneumoniae* have also been associated with human disease (Bochud et al., 1994; Douglas et al., 1993; Keith et al., 2006; Rolo et al., 2013). Hence it is very crucial to identify them accurately for an early diagnosis and better treatment (Roberts et al., 1979; Bochud et al., 1994; Elting et al., 1992; Carratalá et al., 1995). Misidentification of causative agents not only affects the true burden of disease surveillance but also falsely increases the pneumococcal antimicrobial resistance rate since other closely related species such as *S. mitis* are associated with a high rate of penicillin and multidrug resistance (Simões et al., 2010; Wester et al., 2002). In addition, precise identification followed by serotyping is needed for the effective formulation of conjugate vaccine which is based on most prevalent serotypes. Development and implementation of an appropriate vaccine are always driven by proper laboratory identification of the disease causing agents. The current review discusses the different sides of routine culture-based identification of *S. pneumoniae* which involves insensitive bile solubility and optochin susceptibility testing. This review will also consider the uses and limitations of various molecular assays used for identifying and serotyping of *S. pneumoniae*.

2. The role of a conventional method for identification and serotyping

The routine culture-based identification of *S. pneumoniae* involves bile solubility and optochin susceptibility testing (Lund and Henrichsen, 1978). However, these tests are not completely reliable due to the reports that indicate the presence of bile insoluble (2%) (Richter

Abbreviations: CDC, Centre for Disease Control, Atlanta; MALDI-TOF MS, matrix assisted laser desorption/ionization-time of flight mass spectrometry; MLST, multi locus sequence typing; MLSA, multi locus sequence analysis; NCC, null capsule clades; NESp, non-encapsulated *Streptococcus pneumoniae*; NGS, next generation sequencing; NP, nasopharyngeal; ORF, open reading frame; PCR, polymerized chain reaction; PCV, pneumococcal conjugate vaccine; RFLP, restriction fragment length polymorphism; SG/ST, serogroup/serotype; URT, upper respiratory tract; WGS, whole genome sequencing; SM PCR, sequential multiplex polymerized chain reaction

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http://dx.doi.org/10.1016/j.mimet.2017.07.015
Received 21 February 2017; Received in revised form 30 July 2017; Accepted 31 July 2017
Available online 02 August 2017

et al., 2008), optochin resistant strains (10%) of *S. pneumoniae* (Kellogg et al., 2001). Moreover, the presence of similar biochemical properties in a significant proportion of other closely related *Streptococcus* spp. makes it difficult to identify them accurately especially when samples are from respiratory sites (Gray et al., 1980).

Quellung is the gold standard for serotyping, which is based on the reaction between antibodies against the capsule of *S. pneumoniae*. The two Quellung based serotyping methods used are the Danish system and the American system. Serogroup 35A of the Danish system resembles two different American types namely 47 and 62. Therefore one of these two serotypes may be present in 35A which leads to cross reactions (Kauffmann et al., 1960). Those isolates which give inconsistent results with optochin susceptibility, bile solubility and show a negative quellung reaction are referred to as atypical pneumococci. The non-encapsulated strains cannot be serotyped by the Quellung method which are referred to as nonserotypeable *S. pneumoniae* (Ezaki et al., 1988; Pikis et al., 2001; Whatmore et al., 2000). The problem arises when we encounter a pneumococcus that is atypical or nonserotypeable. In such situations, molecular methods play a major role in the identification and diagnosis of pneumococcal disease and its serotyping. Since laboratory isolation of organisms are the major challenge in developing countries where conventional methods require the culture growth of the organism, molecular methods can be used alternatively for even culture negative cases.

3. Role of molecular methods

In the last decade, different molecular assays were developed using an array of pneumococcal specific targets, like - pneumolysin (*ply*) (McAvin et al., 2001), autolysin (*lytA*) (Corless et al., 2001), pneumococcal surface antigen A (*psaA*) (Morrison et al., 2000), manganese-dependent superoxide dismutase (*sodA*) (Kawamura et al., 1999), and penicillin binding protein (*pbp*) (O'Neill et al., 1999) to name a few. But subsequent detection of these pneumococcal targets in viridans group streptococci (Neeleman et al., 2004) were deemed as non-specific targets and therefore unacceptable for routine use in the diagnostic lab. The occurrence of horizontal gene transfer and homologous recombination (Chi et al., 2007; Dowson et al., 1993; Hakenbeck et al., 2001; King et al., 2005) between *S. pneumoniae* and commensal viridans group streptococci gave rise to such genetic similarity between *S. pneumoniae* and other closely related species such as *S. mitis, S. oralis*, and *S. pseudopneumoniae* (Do et al., 2009; Kilian et al., 2008; Whatmore et al., 2000). Details of various molecular methods proposed for identification based on conventional monoplex, multiplex, real-time PCR, and sequencing are listed in Table 1.

Due to extensive genetic exchange in *S. pneumoniae*, no methods were found to be reliable for identification of *S. pneumoniae* and its differentiation from other viridans group streptococci. A combination of molecular and conventional methods should be used to differentiate between nontypeable and atypical *S. pneumoniae* (Kilian et al., 2008; Ing et al., 2012). Recently nontypeable pneumococci were termed as nonencapsulated *S. pneumoniae* (NESp) (Hathaway et al., 2004). NESp is further grouped into either group I or group II based on the presence of the *cps* locus. Group I NESp have *cps* genes, but do not produce capsule due to mutations or deletions in the cps genes. Group II NESp isolates have novel genes in place of the *cps* gene. In these cases the cps locus is replaced by two *aliB*-like homologs called *aliC* and *aliD* (also called aliB ORF1 and ORF 2 respectively) and a gene coding for pneumococcal surface protein K (*pspK*) (Hathaway et al., 2004; Park et al., 2012; Salter et al., 2012). Based on the presence or absence of these three genes, group II is again divided into three null capsule clades (NCCs) (Park et al., 2012). To date, only NCC3 strains are considered as not true pneumococci or closely related streptococcal species (Fig. 1).

Recent studies have reported various molecular methods for the identification and differentiation of *S. pneumoniae* among nontypeable and atypical pneumococcus. These were comprised of PCR followed by RFLP at signature specific positions of *lytA* and 16S rRNA (Llull et al., 2006 and Scholz et al., 2012), partial sequence analysis of *rpoB* and *sodA* (Glazunova et al., 2009; Kawamura et al., 1999), real-time PCR targeting *lytA* gene together with *piaB* (Simões et al., 2016), multi locus sequence typing (MLST) and multi locus sequence analysis (MLSA).

PCR followed by RFLP of signature specific positions of *lytA* and 16S rRNA (Llull et al., 2006 and Scholz et al., 2012) has been proven successful in the identification of *S. pneumoniae*. The partial sequence analysis of *rpoB* (Glazunova et al., 2009) and *sodA* (Kawamura et al., 1999) genes continues to be the ideal tool to identify strains at the species level in the genus streptococcus. The molecular signatures observed in 16SrRNA, *sodA* and *rpoB* genes were identified by Drancourt et al. (2004). Currently, the culture independent method for the detection of pneumococci recommended by the World Health Organization (WHO) is *lyt*A real-time PCR developed by the Center of Disease Control and Prevention (CDC), Atlanta. However, use of *lytA* also could not detect some pneumococcal strains precisely due to presence of a *lytA* homologue in some streptococcal species which also harbor the characteristic pneumococcal *lytA* as *S. pneumoniae*. The identification specificity of *lytA* gene is increased by an additional real-time PCR targeting *piaB* which showed a negative result for *S. pseudopneumoniae* (Simões et al., 2016).

Apart from epidemiological characterization, MLST is used to accurately identify *S. pneumoniae* (Maiden, 2006). This is based on the phylogenetic tree generated using the concatenated sequences of six out of seven housekeeping genes (except *ddl* gene) used for MLST and distinguishing between *S. pneumoniae* and viridans group streptococci (Kilian et al., 2008; Ing et al., 2012). Further, MLSA for viridans group streptococci developed by Bishop et al., uses concatenated sequences of seven housekeeping genes *map*, *pfl*, *ppaC*, *pyk*, *rpoB*, *sodA*, and *tuf* to clearly differentiate Viridans group streptococci up to the species level (Bishop et al., 2009).

Whole genome sequencing (WGS) by Next generation sequencing (NGS) is the latest technology that has been used for a complete characterization of the organism. The presence of mosaicism in genes is high in bacteria like *S. pneumoniae* that undergo extensive recombination (Hakenbeck et al., 2001; Hollingshead et al., 2000; Chi et al., 2007). Mosaicism is most commonly observed in genes that tend to undergo recombination such as penicillin resistance genes (Chi et al., 2007), capsule genes (Trzciński et al., 2004). These should be frequently analyzed through NGS. Whole genome sequence data can be used to retrieve many characteristic features of organisms used to identify *S. pneumoniae* including the full length sequences of various targets such as *lyt*A, 16S rRNA, *sod*A and *rpo*B. WGS helps in the accurate identification of organisms by identifying serotypes (Kapatai et al., 2016), serotype variants (Kamng'ona et al., 2015), antibiotic resistance profile (Metcalf et al., 2016), virulence determinants (Hiller et al., 2011) and vaccine candidates (Wizemann et al., 2001). Additionally, newer antibiotics can be developed by identifying novel genes (Baltz et al., 1998).

4. MALDI-TOF

MALDI-TOF Mass Spectrometry (MS) is considered as a rapid tool for identification of bacteria in diagnostic laboratories (Van Veen et al., 2010). MALDI-TOF can identify the streptococcal genus, but could not differentiate the pneumococci and the mitis group. Recent studies have reported selective peaks in MALDI-TOF-MS pattern of *S. pneumoniae* and *S. mitis* group using ClinProTools™. Ikryannikova et al. (2013) identified a specific peak of 6949 *m/z* in *S. mitis* isolates alone but not seen in *S. pneumoniae* isolates. It was further stated that using three mass peaks (6949, 9876, and 9975 *m/z*) the sensitivity and specificity of identification could be increased close to 100%. Similarly, peak 6955 *m/z* was identified specifically for the non-pneumococcal mitis group species by Werno et al. (2012), who observed that *S. pneumoniae* isolates consistently showed the presence of a correlated peak pair *m/z*