Molecular analysis of *Streptococcus pneumoniae* clones causing invasive disease in children in Singapore

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Streptococcus pneumoniae remains a leading cause of serious paediatric disease. However, there are few published epidemiological data regarding invasive pneumococcal disease (IPD) in many countries in South East Asia, including Singapore. Baseline data for IPD are essential to inform policy regarding pneumococcal conjugate vaccine (PCV) use in Singapore. To our knowledge, this is the first study to use multilocus sequence typing (MLST) to investigate clonal relationships among Singaporean IPD isolates. We characterized 86 invasive pneumococci isolated from Singaporean children between 2001 and 2006 using serotyping and MLST. The objectives were to compare Singaporean MLST data to worldwide data and to assess serotype distribution in relation to current PCV formulations. We observed 50 sequence types (STs), a high proportion of which ($n=16$) were novel STs. Despite the presence of these novel STs, serotype distribution was similar to that observed elsewhere. Serotypes 14, 6B, 19A and 19F accounted for 85 % of IPD cases. PCV7, PCV10 and PCV13 covered 85 %, 86 % and 97 % of IPD isolates, respectively. We have demonstrated a pressing need for larger studies to determine the molecular epidemiology and antibiotic susceptibility of circulating pneumococcal clones from both carriage and disease in Singapore.

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INTRODUCTION

Despite the fact that *Streptococcus pneumoniae* remains a leading cause of pneumonia and invasive disease in children worldwide, there are few published epidemiological data regarding invasive pneumococcal disease (IPD) in South East Asian countries. Existing data regarding the epidemiology of *S. pneumoniae* in Singapore have focussed on disease presentation (Low *et al.*, 2007) or antibiotic resistance (Song *et al.*, 2004a, b). Two previous studies have investigated serotype distribution among Singaporean childhood IPD isolates from 1997–1999 ($n=180$) and 1997–2004 ($n=147$) (Chong *et al.*, 2008; Soh *et al.*, 2000).

Baseline data regarding the serotype prevalence of IPD are essential to inform policy around the use of pneumococcal conjugate vaccines (PCVs) in Singapore. PCV7 has been available on a demand basis in Singapore since October 2005 but was only incorporated into the national childhood vaccination schedule in November 2009 (Vasoo *et al.*, 2010).

Abbreviations: IPD, invasive pneumococcal disease; KKH, Kandang Kerbau Women's and Children's Hospital; MLST, multilocus sequence typing; PCV, pneumococcal conjugate vaccine; ST, sequence type.

Information that aids in understanding the genetic background of pneumococci associated with IPD is also important; the invasive potential of individual clones to succeed and spread geographically is associated with genotype as well as serotype (Hanage *et al.*, 2005; Henriques-Normark *et al.*, 2008; Sandgren *et al.*, 2004). Clonal expansion has been shown to result in notable changes in circulating pneumococcal populations even in the absence of vaccine pressure.

To our knowledge, no published study has used multilocus sequence typing (MLST) to characterize Singaporean pneumococcal disease isolates. The objective of this study was to characterize pneumococci isolated from IPD in Singaporean children using serotyping and MLST and compare these data to data from elsewhere using the MLST database. The secondary objective was to assess the serotype distribution in relation to known PCV formulations.

METHODS

S. pneumoniae was obtained from cases of IPD in children receiving treatment in the Kandang Kerbau Women's and Children's Hospital (KKH), Singapore, between 2001 and 2006. There were 142 isolates

from blood, pleural and cerebrospinal fluid, of which 86 (60.6%) were available for this study. KKH is the only integrated women's and children's hospital in Singapore; it has 830 beds and provides specialized care for women and children. KKH admits children up to 16 years of age.

Serotyping was performed by the Quellung reaction; additionally, serogroup 6 isolates were assigned to serotype 6A, 6B or 6C using the Centers for Disease Control PCR method (da Gloria Carvalho *et al.*, 2009). Genotyping was performed using MLST (Enright & Spratt, 1998) and relationships between clones were defined using eBURST version 3 (Feil *et al.*, 2004). Clonal complexes were defined as groups of sequence types (STs) sharing six or more identical housekeeping alleles. Genotypic diversity among isolates of the same serotype was measured using Simpson's index of diversity (Simpson, 1949). Antibiotic susceptibility was measured using either disc testing (Bell, 1975) (CDS, Australia) or MIC testing (Etest; AB Biodisk). Non-meningitis breakpoints for penicillin were defined according to CDS criteria: penicillin-susceptible, MIC ≤ 0.125 ; penicillin reduced susceptibility, MIC between 0.25 and 2.0; and penicillin-resistant, MIC > 2.0 .

RESULTS

Bacterial isolates

Eighty-six isolates of *S. pneumoniae* were available for use in this study: 77 isolates were from blood, eight from pleural fluid and one from cerebrospinal fluid. Isolates were taken from children aged 2 days to 13 years, mean age 3.68 years (Table 1).

Serotype distribution

Ten different serotypes were detected among the 86 isolates (Table 1). Four serotypes (14, 6B, 19A, 19F) accounted for 85% of IPD cases. Of these, serotype 14 was the most common ($n=35$, 40.7%) followed by 6B ($n=22$, 25.6%), 19A ($n=8$, 9%) and 19F ($n=8$, 9%). No isolates of serotype 6C were observed. The rank order of serotypes in our study was 14, 6B, 19A, 19F and 23F, compared to 14, 6B, 23F and 19F in a recent study by Chong *et al.* (2008). Serotype 23F accounted for only 5/86 (4.65%, 95% CI=0.9–10.8%) of our isolates compared to 15/93 (16%, 95% CI=10–25%) in the Chong *et al.* (2008) study

($P=0.05$). It should be noted that although this study includes strains isolated from 2001 to 2006, only approximately 25% of strains from 2001 and 2002 were available for typing whereas at least 90% of strains isolated during the period from 2004 to 2006 were available. Therefore, this study is skewed for strains isolated later, i.e. from 2004 onwards.

Vaccine and non-vaccine serotypes

Around 85% of the isolates included in the study were of serotypes included in the current seven-valent pneumococcal conjugate vaccine (including vaccine-related serotype 6A but excluding 19A) (Prevenar, PCV7; Wyeth). In our dataset, 84.8% and 96.5% of isolates were of serotypes covered by the recently licensed 10-valent (Synflorix; GSK) and 13-valent (PCV13; Wyeth) formulations. The majority of the increased coverage of PCV13 is due to the inclusion of serotype 19A (9.3% of all isolates).

Clonal relationships between 86 isolates

The 86 pneumococcal isolates included in the study could be divided into 50 different STs, as defined by MLST (Table 1). Using eBURST version 3, the isolates were divided into seven clonal groups and 30 singleton STs (Table 2). ST9 was the most common ($n=11$, 12.8%), ST156 ($n=6$, 7%) was the next most common, followed by ST236 and ST90 (both $n=5$, 5.8%). Serogroup/types that were represented by more than one isolate (6A, 6B, 15, 19A, 23F) demonstrated considerable genetic heterogeneity as defined by MLST. The majority of STs observed in this study were represented by isolates of only one serotype. ST81 and ST490 were the exception to this: ST81 was represented by one isolate each of serotype 19F and 23F and ST490 by two 6B isolates and a serotype 14 isolate.

Of the 34 previously known STs that we observed, 13 have previously been deposited in the database from South East Asian countries (Thailand, Vietnam, Malaysia, China, Japan, Hong Kong, Korea and the Philippines). Five of these STs (ST2757, ST3791, ST2983, ST3790 and ST76) have only been reported from Asia (China, Singapore,

Table 1. *S. pneumoniae* IPD isolates used in the study

Specimen	Patient age group	Serogroup/type	<i>n</i>
Blood	<1 year	6A ($n=2$), 6B ($n=3$), 14 ($n=3$), 19A ($n=2$), 23F ($n=1$)	11
	1–4 years	3 ($n=1$), 6A ($n=2$), 6B ($n=13$), 14 ($n=19$), 19A ($n=1$), 19F ($n=4$), 23F ($n=2$)	42
	5–9 years	6B ($n=5$), 14 ($n=10$), 15 ($n=1$), 19A ($n=2$), 19F ($n=3$), 23A ($n=1$)	22
	10–14 years	1 ($n=1$), 19F ($n=1$)	2
Pleural fluid	1–4 years	6B ($n=1$), 14 ($n=2$), 19A ($n=2$)	5
	5–9 years	14 ($n=1$)	1
Empyema exudate	1–4 years	23 ($n=1$)	1
	5–9 years	19A ($n=1$)	1
Cerebrospinal fluid	1–4 years	15 ($n=1$)	1
Total			86

Table 2. Clonal complexes and associated STs within serogroup/types of Singaporean paediatric IPD isolates

STs identified for the first time in this study are indicated with bold type. STs in square brackets indicate inclusion in a clonal complex (CC).

Serogroup/type	Clonal complex [STs] (no. of isolates)
1	[217] (<i>n</i> =1)
3	[3805] (<i>n</i> =1)
6A	CC1 [1092] (<i>n</i> =1), CC6 [3806] (<i>n</i> =1), 3787 (<i>n</i> =1), 3810 (<i>n</i> =1)
6B*	CC1 [2757/3850/3849] (<i>n</i> =3), CC4 [90/3808] (<i>n</i> =6), CC6 [1518] (<i>n</i> =2), 76 (<i>n</i> =1), 138 (<i>n</i> =1), 146 (<i>n</i> =2), 315 (<i>n</i> =1), 590 (<i>n</i> =2), 2983 (<i>n</i> =2), 3786 (<i>n</i> =1), 3809 (<i>n</i> =1)
14*	CC1 [490] (<i>n</i> =1), CC2 [15/9/13/2562] (<i>n</i> =17), CC3 [200/876] (<i>n</i> =3), 63 (<i>n</i> =1), 124 (<i>n</i> =1), 143 (<i>n</i> =4), 156 (<i>n</i> =6), 554 (<i>n</i> =1), 3785 (<i>n</i> =1), 3790 (<i>n</i> =1), 3803 (<i>n</i> =1)
15	CC5 [1591] (<i>n</i> =1), 3807 (<i>n</i> =1)
19A	CC3 [199] (<i>n</i> =1), 320 (<i>n</i> =2), 1159 (<i>n</i> =1), 1553 (<i>n</i> =1), 2013 (<i>n</i> =1), 230 (<i>n</i> =1), 3789 (<i>n</i> =1)
19F*	CC5 [81] (<i>n</i> =1), 236 (<i>n</i> =5), CC7 [257/3791] (<i>n</i> =2)
23A	338 (1)
23F*	CC5 [81] (1), 242 (2), 311 (1)

*PCV7 serotype.

Hong Kong and Taiwan). We compared the MLST profiles of the 86 Singaporean isolates to the profiles present in the MLST database using eBURST version 3. The majority (*n*=40) of our clones mapped to the main large clonal complexes but 10 STs were singletons not related to any other profile within the database.

Novel STs

Sixteen STs (32 %) were novel STs at the time of analysis. Of these novel STs, nine were novel combinations of alleles, which may have arisen from recombination events, and seven included novel alleles not previously observed (three isolates had novel *gdh* alleles, two had novel *xpt* alleles, one had a novel *ddl* allele and one had a novel *gki* allele). When compared against the STs deposited in the *S. pneumoniae* MLST database (www.mlst.net), the previously reported STs reported here match with serotypes deposited for those STs. Eleven of the 16 novel clones observed in this study were related to (single locus variants of) previously reported STs. The remaining three novel clones were unrelated to any other clones in the MLST database.

Serotype/genotype relationships

Three serotypes (1, 3 and 23A) were represented by only one isolate (therefore one ST); the remaining seven serotypes were all made up from multiple STs. Serotype 6B was the most diverse (Simpson's index of diversity=0.94), with serotype 14 also showing high diversity (Simpson's index of diversity=0.87).

DISCUSSION

Serotype prevalence in our study (isolates collected during 2001–2006) was similar to that found in a recent study of 147 paediatric IPD isolates from KKH during the period

1997–2004 (Chong *et al.*, 2008). However, serotyping data from that study included only strains isolated during 2001–2004. In our study, 63/90 (70 %) pneumococci were isolated during 2004–2006. Only around 25 % of the reported isolates from 2001–2002 were available for inclusion in our study but the 63 pneumococci isolated during 2004–2006 represented over 80 % of isolates reported. Although the same serotypes were found to account for the majority of IPD, the distribution of these serotypes differed slightly in the two studies, the proportion of serotype 23F isolates in our study being more than 10 % less than that observed in the Chong *et al.* (2008) study. This reduction could be indicative of a reduction in prevalence of serotype 23F pneumococci in this region between 1997 and 2006. This is in agreement with other recent data showing a decrease in prevalence of vaccine serotypes causing invasive disease over this time period (our unpublished data). As discussed above, the differences observed between the two studies may also be due in some part to skewing of our data in favour of the later study years. However, a more recent study regarding carriage of pneumococci among children attending day care centres during 2007–2008 found serotype 23F to be among those most commonly carried by children: 6B (16.9 %), 23F (11.9 %) and 19F (10.2 %). Serotype distribution in carriage is not expected to reflect that observed for invasive disease due to the differing propensities of serotypes and genotypes to invade (Brueggemann *et al.*, 2003). Natural fluctuations in the prevalence of pneumococcal serotypes and clones are known to occur (Jefferies *et al.*, 2010; Rückinger *et al.*, 2008); however, larger and more detailed longitudinal studies need to be performed in order to investigate any such fluctuations.

Although the rank order of paediatric IPD serotypes observed in this study was similar to that observed in other studies of childhood IPD (Hausdorff *et al.*, 2000), the clonal composition varied from that observed in the USA and

Europe prior to PCV7 introduction. The most striking example of this was for serotype 14, for which we observed 15 different STs (Table 2). In a UK study carried out in Scotland, serotype 14 was the most common serotype isolated from paediatric IPD prior to PCV7 introduction (Clarke *et al.*, 2006; Jefferies *et al.*, 2010) and was represented by 12 STs of which ST9 and ST124 were by far the most common. In the present study, ST9 was also the most common ($n=11$). We also observed ST124; however, with the exception of ST15 which was found in both datasets, all other serotype 14 isolates observed in Singapore were not observed in the Scottish study and three of the serotype 14 STs observed in Singaporean isolates were novel STs. Serotype 14 frequently displays antibiotic resistance and a recent Taiwanese study demonstrated that environmental drug pressure is the major driving force for genome evolution within this serotype (Ding *et al.*, 2009).

Differences in the clonal composition of common paediatric serotypes are important in the context of IPD, as it is becoming clear that invasive potential is determined not only by serotype but also by genotype (Aguiar *et al.*, 2010; Hanage *et al.*, 2005; Jefferies *et al.*, 2007; Sjöström *et al.*, 2006). Although conjugate vaccines will target the most common serotypes causing IPD in Singapore, capsular switch and subsequent clonal expansion of virulent clones, particularly those resistant to antibiotics, has been observed in the USA in the post-vaccine era (Brueggemann *et al.*, 2007), emphasizing the need for surveillance at the genotypic level.

Of the 50 pneumococcal clones (STs) observed in this study, 16 (32%) had not been previously reported. Although these 16 STs have not been previously reported, this gives no indication of their prevalence within this region as pneumococci from Singapore and other South East Asian countries are under-represented on the MLST database. Clonal properties associated with these STs, including invasive potential, are therefore unknown. The presence of new clones circulating in South East Asia underlines the importance of further epidemiological characterization of pneumococci in this region. Twenty-one of the clones that we identified during the study (including the 16 new clones) have only been reported to the MLST database from South East Asian countries. Whilst this does not preclude the existence of these clones elsewhere, the presence of such STs may indicate that particular clones, uncommon in other parts of the world, are emerging in this region in the absence of widespread PCV use.

Ninety-seven per cent of isolates in our study were PCV13 serotypes, while around 85–86% of isolates were PCV7 and PCV10 serotypes. This difference is largely attributable to the prevalence of serotype 19A, which accounted for 9.3% of our isolates. This figure differs from the 2% prevalence of serotype 19A in the dataset reported by Chong *et al.* (2008). The prevalence of non-vaccine serotype IPD, and, in particular, IPD due to serotype 19A, has been increasing

in other countries in recent years. Such increases may be due to selection pressure after widespread use of PCV7 (Hicks *et al.*, 2007; Moore *et al.*, 2008; Pai *et al.*, 2005) and is also reflected in carriage isolates (Hanage *et al.*, 2007). However, increases in 19A IPD have also been observed prior to the introduction of PCV7 (Jefferies *et al.*, 2010). Together with laboratory data (Lee *et al.*, 2009), these findings indicate that there is little or no cross-protection against 19A pneumococci from 19F polysaccharide. Of the two increased-valency PCVs, PCV13 includes serotype 19A whereas PCV10 does not. PCV7 has been available in Singapore since 2005 and is available on demand in the private market. In September 2009, the Singapore Ministry of Health approved PCV7 for inclusion into routine childhood vaccination. PCV10 and PCV13 are not currently licensed for use in Singapore.

A multi-resistant 19A ST320 isolate was identified during this study; this isolate displayed penicillin Etest MICs greater than $1.0 \mu\text{g ml}^{-1}$ and was also resistant to erythromycin and co-trimoxazole (data not shown). As discussed above, there is little cross-protection against 19A pneumococci from 19F polysaccharide and increases in 19A disease have occurred in some countries post PCV7. However, such increases may not be driven entirely by vaccine pressure; a recent study from Korea demonstrated that expansion of a multidrug-resistant (MDR) ST320 variant of the clone observed in our study was responsible for a large increase in serotype 19A incidence, prior to vaccine introduction (Choi *et al.*, 2008). The presence of MDR ST320 in Singapore warrants close surveillance of pneumococcal molecular epidemiology at a national level. Awareness of pneumococcal disease is increasing in South East Asia; genotyping using molecular techniques such as MLST will aid in charting the spread of invasive pneumococcal clones in the region and allow comparison with different geographical areas.

Our study includes only a relatively small number of isolates from one geographical area of Singapore; therefore conclusions regarding serotype coverage for the three conjugate vaccines cannot be drawn. However, such vaccines have the potential to successfully reduce morbidity and mortality from pneumococcal disease. Therefore, we have demonstrated a pressing need for larger studies to determine the molecular epidemiology and antibiotic susceptibility of circulating pneumococcal clones from both carriage and disease in Singapore and other South East Asian countries. Enhanced surveillance is required in order to capture information regarding pneumococcal population dynamics prior to widespread use of PCV7 so that any changes in the post-vaccine era can be appropriately analysed. Informed decisions regarding vaccine policy can only be made in the light of such studies.

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