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Commentary: Comparative Genomic Analysis of Ten Clinical *Streptococcus pneumoniae* Collected From a Malaysian Hospital Reveal 31 New Unique Drug-Resistant SNPs Using Whole Genome Sequencing

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Abstract

Despite the effort and decades of research, *S. pneumoniae* remains a primary cause of infectious morbidity and mortality worldwide. Although Antibiotics are lifesaving medications that offer tremendous benefits to patients with infectious diseases. Yet, several reports have revealed that the overuse and misuse of these agents had led to antibiotic resistance. Our study utilized whole genome sequencing (WGS) to reveal the pattern of antibiotic-resistance among ten pneumococcal isolates with various degree of susceptibility to antibacterial drugs. The main purpose of our study was to explore genetic variations related to drug-resistance in those ten strains. The results indicated that pneumococcal strains with resistant profile were associated with greater number of SNPs compared to susceptible ones. Out of all the SNPs identified, 31 were unique and had not been reported before. Our data propose that these SNPs could possess an important role in modifying the degree of sensitivity to different antibacterial drugs. In this article we comment on the methodology and results of our study which previously published in Journal of Biomedical Science.

Introduction

Streptococcus pneumoniae or the pneumococcus is a major cause of community-acquired pneumonia and meningitis, as well as bloodstream, ear, and sinus infections[1–4]. Globally, it is estimated that this bacterial pathogen colonizes as many as 40–60% of young children. While colonization most often results in asymptomatic carriage, *S. pneumoniae* is still responsible for a substantial burden of disease[5]. In 2000, pneumococcus caused 14.5 million episodes of severe pneumococcal infections resulting in 826 000 deaths in children beneath the age of five[6]. Apart from pneumococcal deaths in HIV-positive children, death caused by pneumococcus accounts for approximately 11% of under-five mortality[5]. Decades of overuse of antibiotics in medical and agricultural applications as well as inappropriate prescribing of these drugs were the primary driver of antibiotic resistance crisis[7–9]. Drug-resistant S. pneumoniae (DRSP) has become an important clinical and public health problem during the past 20 years[10]. Pneumococcal resistance to different antibiotics led to 32,398 extra outpatient visits and 19,336 additional hospitalizations, accounting for $91 million (4%) in direct medical costs and $233 million (5%) in total costs, including work and productivity losses[11]. In order to overcome this issue and for better understanding on how pneumococci develop resistance to different types of antibiotics we

utilized Whole Genome Sequencing (WGS) in our study[12]. WGS allow researchers to study the mode of action of antibiotics and the mechanisms involved in bacterial resistance[13,14]. Also, WGS can be applied by scientists to investigate the molecular basis and rate of evolution of antibiotic resistance in real-time under treatment regimens of single drugs or drugs combinations[15]. In our study, we used Whole Genome Sequencing to reveal the patterns of resistance of 10 pneumococcal isolates with a range of susceptibility and resistance to four different antibiotics: penicillin, cefotaxime, erythromycin, and tetracycline. The aim of our study was to investigate the genetic variation among pneumococcal isolates with different susceptibility profiles to four antibiotics in order to identify SNPs associated with virulent genes that could be a target for drug development.

Methodolgy

The aim of our study is to identifiy Single Nucleotide Polymorphisms (SNPs) that are associated with antibiotic-resistance. Association of a SNP with drug resistance implicates genes that either reside near the genomic location of the SNP, or are regulated by a genetic factor located there. Ten clinical isolates of *S. pneumoniae* were collected previously from University of Malaya Medical Centre (UMMC) (Table 1). The genomes of *S. pneumoniae* clinical isolates were extracted using DNeasy Blood & Tissue Kit (Qiagen), the quantity and purity of the DNA was measured using qubit (Table 2). DNA fragmentation was done using Covaris S2. The fragmented DNA were ends repaired, added with dA base and ligated with Illumina indexed adapters. Standard concentration was used as the quantification becomes less reproducible, the sequencing library becomes less stable and subsequently Lower sequencing yield is the likely outcome. Size selections of the samples were performed using Invitrogen 2% agarose E-gels. The selected DNA fragments with adapters molecules on both ends underwent 10 cycles of PCR for amplification of prepared material. The samples were then diluted to 10Nm using hybridization buffer and pooled in to one pool. The libraries were loaded onto 1 lane of Illumina HiSeq 2000 flow cell v3 for sequencing.

Table 2: Quantity of Samples using Qubit

Sample ID	ng/ul	Volume (ul)	Total ng
SPS1	80.3	80	6424
SPS2	237	80	18960
SPS3	105	80	8400
SPS4	78.3	80	6264
SPS5	172	80	13760
SPS6	127	80	10160
SPS7	70.6	80	5648
SPS8	70.8	80	6372
SPS9	64.7	90	5823
SPS10	31.6	90	2844

In order to exclude low quality reads, PRINSEQ version 0.20.3 was used and the following types of reads were removed:

1. Reads having 'N' in more than 10% of the total bases of that read
2. Reads with Phred quality score less than 20.
3. Reads shorter than 50 bp.

In order to evaluate the core genome average identities and completeness, the sequenced reads were assembled and mapped against *S. pneumoniae* TIGR4. SPAdes assembler was used in our study to assemble the genomic DNA extracted from the bacterial samples. This software is initially designed to assemble small genomes from MDA single-cell and standard bacterial data sets. Assembly of single cell data is challenging due to non-uniform read coverage, difference in insert length, high levels of sequencing errors and chimeric reads. Thus, SPAdes addresses these issues by performing assembly in four stages:

1. SPAdes proposes a new approach to assembly graph construction that uses the *multisized de Bruijn graph*, implementation of new bulge/tip removal algorithms, detection and removing of

Table 1: Bacterial strains and sources used for the genomic comparison of *S. pneumoniae* strains.

Isolate	Isolation date	Sex	Source	Serotype[a]
SPS1	15/9/2010	NA	Nasopharyngeal swab	NT
SPS2	21/5/2011	Female	Nasopharyngeal swab	1
SPS3	21/5/2011	Male	Nasopharyngeal swab	19F
SPS4	20/2/2012	Female	Nasopharyngeal swab	14
SPS5	16/3/2012	Female	Swab from eye	23F
SPS6	18/5/2012	Male	Nasopharyngeal swab	15B/C
SPS7	9/5/2011	Male	Blood	1
SPS8	8/3/2011	Female	Nasopharyngeal swab	14
SPS9	26/4/2011	Male	Blood	18
SPS10	10/5/2011	Male	Blood	8

[a] all serotypes were identified using multiplex PCR as described before (Pai et al., 2006).

Abbreviations: NA, not available; NT, non-typeable.

chimeric reads, aggregation of biread information into *distance histograms*, and allowing of backtrack the performed graph operations.

2. *k*-bimer adjustment, SPAdes derives accurate distance estimates between *k*-mers in the genome using joint analysis of distance histograms and paths in the assembly graph.

3. Paired assembly graph construction. By using *k-bimer adjustment* approach, SPAdes first extracts *k*-bimers from bireads, resulting in *k*-bimers with inexact distance estimates. The second step is transforming this set of *k*-bimers into a set of adjusted *k*-bimers with exact or almost exact distance estimates.

Table 3: Antibiotic susceptibility profiles of *S. pneumoniae* isolates.

Isolate [a]	MIC (µg/ml)[b]			
	PEN [c]	CTX [c]	ERY [c]	TET [c]
SPS1	2	1	2	16
SPS2	2	1	>2	>16
SPS3	4	1	>2	>16
SPS4	0.06	≤0.063	≤0.016	>16
SPS5	1	0.125	0.031	4
SPS6	0.06	≤0.063	≤0.016	≤0.125
SPS7	2	2	>2	>16
SPS8	0.5	>8	2	>16
SPS9	0.25	8	2	16
SPS10	2	2	>2	16

[a] Isolates SPS1, SPS2, and SPS3 are non-susceptible to all antibiotics. Isolate SPS4 is susceptible to penicillin, cefotaxime, and erythromycin, but resistant to tetracycline. SPS5 is susceptible to cefotaxime and erythromycin, but resistant to penicillin and tetracycline. SPS6 is susceptible to all four antibiotics. SPS7 and SPS10 are resistant to all four antibiotics, SPS8 and SPS9 were resistant to all antibiotics except penicillin.

[b] MIC, Minimum inhibitory concentration.

[c] PEN, Penicillin; CTX, Cefotaxime; ERY, Erythromycin; TET, Tetracycline.

4. Contig construction.

To build a phylogenetic tree based on the identified SNPs, kSNP3 program was used. The reason for using this software over other available ones is that kSNP detects SNPs and builds phylogenies for large numbers of finished and draft sequences. Unlike other methods such as Parsnp which aligns the core genome and requires finished or assembled genomes, kSNP can use raw reads and is able to analyze hundreds of bacterial or viral genomes in only a few hours. In addition, kSNP can build Maximum Likelihood, Neighbor Joining, and parsimony phylogenetic trees based on all SNPs, only core SNPs, and SNPs present in at least a user-specified fraction of genomes. Realphy is another method to build a phylogenetic tree. This method maps raw reads to several reference genomes, therefore increasing the probability of using all of the information in the raw-read genomes for analysis. However, this method relays on accurate mapping of raw reads to the reference genomes, and if some taxa are diverged by > 5–10% the distances to the reference genome are under estimated, leading to incorrect topologies. kSNP overcomes this issue By not relaying on reference genome and by the ability of using raw read files.

Results: 31 Unique SNPs Associated with Virulent Genes were Identified

Ten pneumococcal isolates with different sensitivity to four antibiotics were used in this study (Table 3). By using WGS we were able to found that the majority of the non-synonymous SNPs associated with pneumococcal essential genes were present in antibiotic resistant strains [12]. Through our analysis we were able to identify 90 non-synonymous SNPs related to the essential genes of the resistant strains, and some of them have reappeared in more than one resistant isolate, while none of these SNPs have occurred in susceptible isolates (Table 4). In addition, we were able to

Table 4: Conserved non-synonymous Single Nucleotide Polymorphisms (SNPs) associated with Penicillin Binding Proteins (PBPs) and other virulent genes found in resistant isolates.

Locus Name	Putative Identification	Reference Position	TIGR4	SNP	Pneumococcal isolate	Amino Acid Change
SP_0346	cpsA; capsular polysaccharide biosynthesis protein	320234	C	T	SPS7, SPS8, SPS10	A53V
		320204	C	T	SPS7, SPS8	A43V
		320872	C	T	SPS10	P266S
		321451	G	A	SPS10	V459M
		320582	C	T	SPS10	A169V
		320657	C	T	SPS9, SPS10	S194L
		320560	A	G	SPS10	N162D
		321410	T	C	SPS9	M445T
		320314	G	C	SPS1, SPS9	V80L
		321460	A	G	SPS2	I462V
		321485	T	C	SPS7, SPS8	V470A
		320101	C	A	SPS2	R9S
		320710	A	G	SPS1	T212A

SP_0347	cpsB; capsular polysaccharide biosynthesis protein Cps4B	321798	A	G	SPS2, SPS10	E92G
		321954	A	G	SPS10	E144G
		322094	G	A	SPS10	D191N
		322169	C	T	SPS10	L216F
		322235	G	A	SPS10	V238I
		321579	G	T	SPS9	R19I
		321608	T	G	SPS1, SPS2, SPS7, SPS8	S29A
SP_0348	cpsC; capsular polysaccharide biosynthesis protein	322306	G	T	SPS2, SPS10	V15F
		322313	G	C	SPS10	S17T
		322321	A	G	SPS10	K20E
		322342	A	T	SPS2, SPS10	I27L
		322456	C	T	SPS10	P65S
		322489	A	T	SPS10	T76S
		322853	A	T	SPS1, SPS7, SPS8	H197L
		322360	G	A	SPS2	G33S
		322693	G	A	SPS7, SPS8	E144K
		322549	G	A	SPS9	V96I
SP_0349	cpsD; capsular polysaccharide biosynthesis protein	323314	G	A	SPS9	V117I
		323202	G	A	SPS9	M79I
		323488	G	A	SPS1, SPS7, SPS8	V175I
		323191	A	C	SPS1, SPS7, SPS8	N76H
		323193	T	A	SPS1, SPS7, SPS8	N76K
		323416	A	G	SPS1, SPS7, SPS8, SPS9	I151V
SP_1837	capsular polysaccharide biosynthesis protein	1746914	T	C	SPS1, SPS9, SPS10	K212R
		1747016	A	G	SPS1, SPS2, SPS7, SPS8, SPS9, SPS10	I178T
		1747484	A	G	SPS1, SPS2, SPS7, SPS8, SPS9, SPS10	V22A
		1747494	T	C	SPS1, SPS2, SPS7, SPS8, SPS9, SPS10	T19A
SP_0117	pspA; pneumococcal surface protein A	118489	A	G	SPS1, SPS7, SPS8, SPS9	T23A
		118490	C	T	SPS9	T23M
		120628	A	G	SPS9, SPS10	K736E
		120431	C	A	SPS7, SPS8	A670D
		119178	A	C	SPS7, SPS8	K252N
		119449	A	C	SPS7, SPS8	K343Q
		119056	T	G	SPS1	Y212D
		118496	A	C	SPS7, SPS8, SPS10	Q25P
SP_0799	ciaH; sensor histidine kinase ClaH	753163	C	G	SPS7, SPS8	H180D
SP_1923	pln; pneumolysin	1832851	G	A	SPS9	T154M
		1832174	T	C	SPS2, SPS9, SPS10	N380D
		1832641	T	C	SPS10	K224R
		1832797	G	A	SPS10	T172I
		1831975	G	A	SPS7, SPS8	P446L
		1832906	G	T	SPS1, SPS7, SPS8	Q136K
SP_1937	lytA; autolysin	1840479	T	G	SPS2	L295I
		1840604	A	T	SPS2	E253V
		1840608	C	T	SPS2	N252D
		1840473	A	G	SPS9	P297S
		1840624	A	C	SPS1	D246E
SP_0369	penicillin-binding protein 1A	347449	C	T	SPS1	A522T
		347857	C	T	SPS1	V386I
		347479	C	T	SPS2	E512K
		348706	T	A	SPS2	T103S
		347473	C	G	SPS10	E514Q

SP_2099	penicillin-binding protein 1B	2006807	A	G	SPS10	V787A
		2007578	T	G	SPS9	E530A
SP_2010	penicillin-binding protein 2A	1917863	T	C	SPS9, SPS10	E17G
		1917045	T	C	SPS9, SPS10	T290A
		1916273	C	T	SPS1, SPS9, SPS10	S547N
		1916459	T	G	SPS9	A485E
		1916166	C	T	SPS9	A583T
		1917111	G	T	SPS2	Q268K
		1916595	C	T	SPS2	D440N
		1916819	A	G	SPS1	F365S
SP_1673	penicillin-binding protein 2B	1573249	C	T	SPS7, SPS8, SPS9, SPS10	G597E
		1573212	C	A	SPS9	L609F
		1574933	C	T	SPS3	V36I
		1573493	C	A	SPS2	A516S
		1574288	C	A	SPS2, SPS3	A251S
		1574461	G	A	SPS2	A193V
SP_0336	penicillin-binding protein 2X	309007	C	T	SPS9, SPS10	L710F
		308341	G	A	SPS9	D488N
		307393	G	A	SPS2, SPS3	A172T
		309113	C	A	SPS3	T745K
SP_0798	ciaR; DNA-binding response regulator	751980	G	A	SPS2	V7I
SP_0377	cbpC; choline-binding protein C	356412	G	A	SPS1	G156S
		355972	A	C	SPS2	Q9P
		355974	G	A	SPS2	V10I
		356806	C	T	SPS2	S287L
		356182	C	A	SPS7, SPS8	P79H
		356044	A	G	SPS10	R33Q

identify 31 unique SNPs associated with penicillin binding proteins, pneumolysin, PspA, sensor histidine kinase (ciaH) and capsular polysaccharide biosynthesis protein CpsA (Table 5). Phylogenetic analysis is the most commonly used tool to predict biological relationships. We used the parsimony tree to estimate the phylogenetic relationships among the clinical strains of *S. pneumonaie*. Our results are in agreement with the MIC profile of the ten pneumococcal

Table 5: Unique non-synonymous Single Nucleotide Polymorphisms (SNPs) associated with Penicillin Binding Proteins (PBPs) and other virulent genes found in all ten pneumococcal isolates isolates.

Locus Name	Putative Identification	Reference Position	TIGR4	SNP	Pneumococcal isolate	Amino Acid Change
SP_0346	cpsA; capsular polysaccharide biosynthesis protein	320234	C	T	SPS7, SPS8, SPS10	A53V
		320204	C	T	SPS7, SPS8	A43V
		320872	C	T	SPS10	P266S
		321451	G	A	SPS10	V459M
		320657	C	T	SPS9, SPS10	S194L
		321460	A	G	SPS2	I462V
		321485	T	C	SPS7, SPS8	V470A
		320710	A	G	SPS1	T212A
		322321	A	G	SPS10	K20E
		322360	G	A	SPS2	G33S
		323191	A	C	SPS1, SPS7, SPS8	N76H
SP_1837	capsular polysaccharide biosynthesis protein	1746914	T	C	SPS1, SPS9, SPS10	K212R
SP_0117	pspA; pneumococcal surface protein A	118490	C	T	SPS9	T23M
		120431	C	A	SPS7, SPS8	A670D
		118496	A	C	SPS7, SPS8, SPS10	Q25P
SP_0799	ciaH; sensor histidine kinase ClaH	753163	C	G	SPS7, SPS8	H180D
SP_1923	pln; pneumolysin	1832851	G	A	SPS9	T154M

SP_0369	penicillin-binding protein 1A	347479	C	T	SPS2	E512K
		348706	T	A	SPS2	T103S
SP_2099	penicillin-binding protein 1B	2006807	A	G	SPS10	V787A
SP_2010	penicillin-binding protein 2A	1917863	T	C	SPS9, SPS10	E17G
		1916459	T	G	SPS9	A485E
		1916166	C	T	SPS9	A583T
SP_1673	penA; penicillin-binding protein 2B	1573212	C	A	SPS9	L609F
		1574933	C	T	SPS3	V36I
		1573493	C	A	SPS2	A516S
		1574288	C	A	SPS2, SPS3	A251S
		1574461	G	A	SPS2	A193V
SP_0336	penicillin-binding protein 2X	308341	G	A	SPS9	D488N
		307393	G	A	SPS2, SPS3	A172T
		309113	C	A	SPS3	T745K

strains. The observations that pneumococcal isolates with similar MIC profile were gathered together in the phylogenetic tree propose that these strains possess shared mutations and were probably originated from the same clone. It is possible that these strains could have evolved and acquired mutations in a similar manner due to selection pressures. The high phylogenetic relatedness among the clinical pneumococcal isolates with similar MIC profile is related to the specific SNPs in the mutated genes. The presence of identical uncommon mutations, as well as certain genes in the grouped isolates in the phylogenetic tree, is indicative of a single cluster of strains circulating in the population.

Conclusion

In summary, we compared the genomic sequences of ten pneumococcal strains isolated from University of Malaya Medical Centre (UMMC) with different sensitivity to four different antibiotics: penicillin, cefotaxime, erythromycin, and tetracycline in order to identify the genetic variations within the sequences of these isolates and identifying SNPs that could play significant role in conferring resistance to those antibiotics. The high level of sequence conservation and the presence of the same mutations mainly those associated with genes involved in β-lactam resistance in both sensitive and resistant isolates makes it a difficult task to identify distinct mechanisms of resistance that differentiate strains with different drug-sensitivities, and that antibiotic resistance cannot be only linked to the presence of certain genes. Nevertheless, through our extensive analysis we were able to identify unique SNPs associated with virulent genes that could play a key role in resistance to various antibiotics. However, the small number of the clinical samples included in this study has limited our understanding to the role of these SNPs in conferring resistance toward different antibiotics. Moreover, all resistant genes have yet to be subjected to individual mutational analysis. This can be achieved by introducing the identified SNPs to the resistant genes by site-directed mutagenesis and further expression analysis to confirm the role of these SNPs in conferring antibiotic resistance.

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