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Whole Genome Sequencing Analysis of Penicillin-Susceptible, -Intermediate and -Resistant *Streptococcus pneumoniae* Isolated in Canada, 2011-2016

R.K. HINK[1], A.R. GOLDEN[1], H.J. ADAM[1,2], I. MARTIN[3], W. DEMCZUK[3], S. PETERSON[3], K.A. NICHOL[2], P. VAN CAESEELE[1,4], G.J. GERMAN[5], B. LEFEBVRE[6], G. ZAHARIADIS[7], J.B. GUBBAY[8], D. HALDANE[9], J. MINION[10], R. GAD[11], M.R. MULVEY[1,3], J.A. KARLOWSKY[1,2], G.G. ZHANEL[1] and the Canadian Antimicrobial Resistance Alliance (CARA)

[1]University of Manitoba, [2]Shared Health, [3]National Microbiology Laboratory and [4]Cadham Provincial Laboratory, Winnipeg, MB, Canada; [5]Health PEI, Charlottetown, PEI, Canada; [6]Laboratoire de Santé Publique du Quebec, Ste-Anne-de-Bellevue, QC, Canada; [7]Newfoundland Public Health Laboratory, St. John's, NLA, Canada; [8]Public Health Ontario, Toronto, ON, Canada; [9]Queen Elizabeth II Health Science Centre, Halifax, NS, Canada; [10] Roy Romanow Provincial Laboratory, Regina, SK, Canada; [11] New Brunswick Department of Health, Fredericton, NB, Canada.

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University of Manitoba

Rachel Hink
Microbiology, Health Sciences Centre
MS673-820 Sherbrook St.
Winnipeg, MB R3A 1R9
Email: umhinkr@myumanitoba.ca

Introduction

Streptococcus pneumoniae is a clinically important bacterial pathogen known to cause invasive infections such as bacteraemia and meningitis[1,2]. Despite the availability of pneumococcal vaccines, invasive pneumococcal disease (IPD) continues to cause significant morbidity and mortality globally[3].

β-lactams (e.g., penicillin, ceftriaxone) are commonly use to treat IPD when an isolate is susceptible[1,4]. β-lactams selectively bind and inhibit penicillin binding proteins (PBPs), thereby preventing peptidoglycan polymerization and cell wall synthesis[1,5]. The penicillin binding region of PBPs contain the conserved SXXK, S/YXN and K/H(S/T)G active site motifs[5,6]. To date, six unique PBP types have been identified in *S. pneumoniae* and are classed on the basis of structure and function: PBP1a, 1b, and 2a (class A); PBP2x and 2b (class B); and PBP3[5,6].

Resistance to β-lactams in *S. pneumoniae* is attributed to highly altered PBPs that exhibit decreased β-lactam affinity[1,5,6]. The accumulation of alterations is a result of frequent homologous recombination events between *S. pneumoniae* and related *Streptococcus* spp[1,5,6]. Of the six PBP types, PBP2x, 2b and 1a are commonly altered in clinical isolates of *S. pneumoniae* with reduced susceptibility or resistance to β-lactams[1,5].

The purpose of the present study was to use whole genome sequencing (WGS) to characterize penicillin-susceptible, -intermediate and -resistant invasive isolates of *S. pneumoniae* collected in Canada from 2011 to 2016.

Materials and Methods

Bacterial Isolates: Between 2011 and 2016, a total of 7622 invasive *S. pneumoniae* isolates were submitted to the Public Health Agency of Canada – National Microbiology Laboratory (PHAC-NML) by provincial public health laboratories. As part of the SAVE study, carried out in collaboration between CARA (Health Sciences Center, Winnipeg, Canada) and PHAC-NML, submitted isolates were forwarded to CARA from participating CPHLN sites. A subset of 196 isolates was selected for WGS based on their penicillin MIC; 10% of all isolates for each penicillin MIC value, ranging from 0.06 to 4 µg/mL, were randomly selected for WGS. The remaining isolates were randomly selected from isolates with penicillin MICs of 0.001-0.03 µg/mL.

Phenotypic Characterization: Antimicrobial susceptibility testing was performed by the CLSI broth microdilution method[7]. MICs were interpreted using CLSI MIC breakpoint criteria[8]. Isolates were serotyped by the Quellung reaction using pool, group, type and factor specific antisera (Statens Serum Institute, Copenhagen, Denmark).

WGS Analysis: WGS was performed using the Illumina MiSeq platform. Read quality was assessed by FastQC v2.3; reads with <10x coverage were removed from subsequent analysis. High quality reads were assembled into contigs using SPAdes v1.6 (n=189). The nucleotide and amino acid sequences of PBP2x, PBP2b and PBP1a were annotated using Prokka v1.1 and aligned using ClustalW. Pair-wise distances for each PBP type were calculated using MEGA X and clustal alignments by the p-distance method. Neighbor-joining trees of select *pbp2x* sequences were generated with MEGA X and clustal alignments from a subset of isolates (n=50) using standard parameters and 500 bootstrap replicates. Alterations in PBP active site motifs were identified using Aliview v1.25.

Results

Table 1. Comparison of the number of single nucleotide variants (SNVs) and amino acid substitutions in *S. pneumoniae* PBP2x, PBP2b and PBP1a sequences by penicillin MIC

Penicillin Interpretive Category[a]	Penicillin MIC (µg/mL)	PBP2x			PBP2b			PBP1a		
		Average No. SNVs[b]	SNV p-distance[c] (%)	Average No. substitutions	Average No. SNVs	SNV p-distance (%)	Average No. substitutions	Average No. SNVs	SNV p-distance (%)	Average No. substitutions
R	≥4	294	13.1	70	217	10.6	48	404	18.7	76
	2	301	13.4	72	165	8.1	35	348	16.1	70
I	1	324	14.4	76	121	5.9	23	365	16.9	77
	0.5	275	12.2	63	94	4.6	18	206	9.6	40
	0.25	195	8.7	37	105	5.1	23	120	5.6	27
	0.12	169	8.0	34	108	5.2	22	91	4.2	22
S	0.06	102	4.5	22	36	1.8	7	36	1.7	12
	≤0.03	9	0.4	2	8	0.4	2	11	0.5	4

[a] CLSI oral penicillin V breakpoints (Susceptible=≤0.06 µg/mL, Intermediate=0.12-1 µg/mL, Resistant=≥2 µg/mL)
[b] Averaged from isolate total per MIC value.
[c] Percent nucleotide difference compared to *Streptococcus pneumoniae* R6 reference *pbp2x, pbp2b,* and *pbp1a* sequences.

Figure 1. Neighbor-joining tree of select *S. pneumoniae pbp2x* sequences. Branches are colored by CLSI oral penicillin V breakpoints. Branch information includes serotype (ST) and penicillin (PEN) MIC (µg/mL).

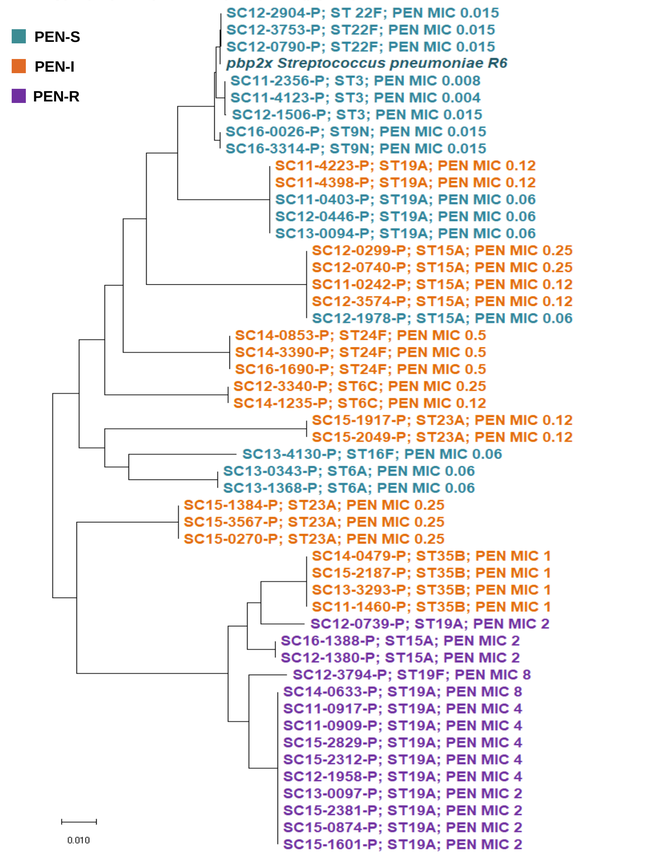

Table 2. Alterations in PBP2b active site motifs of penicillin-susceptible, -intermediate and -resistant isolates of *S. pneumoniae*

Penicillin Interpretive Category[a]	Penicillin MIC (µg/mL)	No. of Isolates	PBP2b		
			SVVK	SSNT	KTGTA
R	≥4	11	----	---A	----G
	2	6	----	---A	-----
		5	----	---A	----G
I	1	8	----	---A	-----
		1	----	---A	----G
	0.5	8	----	---A	-----
	0.25	15	----	---A	-----
		1	----	----	-----
	0.12	25	----	---A	-----
S	0.06	22	----	----	-----
		14	----	---A	-----
	≤0.03	73	----	----	-----

[a] CLSI oral penicillin V breakpoints

Table 3. Alterations in PBP1a active site motifs of penicillin-susceptible, -intermediate and -resistant isolates of *S. pneumoniae*

Penicillin Interpretive Category[a]	Penicillin MIC (µg/mL)	No. of Isolates	PBP1a		
			STMK	SRNVP	KTG
R	≥4	11	-S--	----T	---
	2	7	-S--	----T	---
		3	-A--	----T	---
		1	-S--	-----	---
I	1	5	-S--	-----	---
		3	-A--	----T	---
		1	-S--	----T	---
	0.5	4	-A--	----T	---
		2	-S--	-----	---
		2	----	-----	---
	0.25	11	----	-----	---
		4	----	----T	---
		1	-A--	----T	---
	0.12	23	----	-----	---
		1	----	----T	---
		1	-A--	-----	---
S	0.06	36	----	-----	---
	≤0.03	73	----	-----	---

[a] CLSI oral penicillin V breakpoints

Conclusions

1. Overall, PBP2x, PBP2b and PBP1a were largely unaltered in penicillin-susceptible isolates with MICs ≤0.03 µg/mL, demonstrating ≤0.5% nucleotide difference compared to penicillin susceptible *S. pneumoniae* R6.
2. For *S. pneumoniae* isolates with penicillin MIC values ranging from 0.06-0.25 µg/mL, the rank order of the average number of SNVs among PBP types was: *pbp2x* > *pbp2b* ≈ *pbp1a*. The rank order shifts to *pbp2x* > *pbp1a* > *pbp2b* in isolates with a penicillin MIC of 0.5 µg/mL.
3. For *S. pneumoniae* isolates with penicillin MICs ≥1 µg/mL, the rank order of the average number of SNVs among PBP types was: *pbp1a* > *pbp2x* > *pbp2b.*
4. According to the Neighbor-Joining method:
   - *pbp2x* sequences cluster independently of serotype in penicillin-susceptible isolates with MICs ≤0.03 µg/mL.
   - For isolates with penicillin MIC values ranging from 0.06-1 µg/mL, *pbp2x* sequences cluster largely by serotype.
   - A single cluster containing *pbp2x* sequences of penicillin-resistant isolates (≥2 µg/mL) was observed.
5. A Thr451Ala substitution in the SSNT motif of PBP2b was identified in 80.2% (93/116) of *S. pneumoniae* isolates with penicillin MICs ≥0.06 µg/mL. This substitution was observed in combination with an A619G substitution in the KTGTA motif in 72.7% (16/22) of penicillin-resistant isolates (≥2 µg/mL).
6. A T371A/S substitution in the STMK motif of PBP1a was identified in 94.9% (37/39) of *S. pneumoniae* isolates with penicillin MICs ≥0.5 µg/mL. This substitution was observed in combination with a P432T substitution in the SRNVP motif in 95.5% (21/22) of penicillin-resistant isolates (≥2 µg/mL).
7. A T337A substitution in the STMK motif of PBP2x was identified in 59.5% (69/116) of *S. pneumoniae* isolates with penicillin MICs ≥0.06 µg/mL. This substitution was observed in combination with a L546V substitution in the LKSGT motif in 100% (22/22) of penicillin-resistant isolates (≥2 µg/mL, data not shown).

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