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Characterization by Whole Genome Sequencing of *Streptococcus pneumoniae* Causing Invasive Infections in Canada, 2011-2014

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ABSTRACT

Background: *Streptococcus pneumoniae* (SPN) is a highly diverse organism capable of causing invasive disease. The goal of this study was to characterize a subset of invasive SPN isolates collected in Canada utilizing whole genome sequencing (WGS), specifically by thoroughly investigating putative capsular switches and the national distribution of multi-drug resistant (MDR) strains.

Methods: In a collaboration between CARA and NML, 5012 invasive SPN isolates were collected from across Canada from 2011-14. Serotyping was performed by the Quellung reaction and susceptibility testing was performed using CLSI methods. MDR was defined as resistance to ≥3 classes of antimicrobials. A subset of isolates (the ten most common serotypes and new PCV-15 inclusions: 7F, 19A, 22F, 3, 12F, 11A, 8, 15A, 9N and 6C, plus 33F) were characterized by PFGE/MLST. WGS was performed on 162 isolates using the Illumina MiSeq platform; of these, 84 isolates were selected due to previous characterization that indicated MDR, novel MLST sequence types and/or the potential to be a capsular switch variant. To achieve broader coverage of the population, 78 additional isolates were selected randomly as the "background". To help discern the evolution of strains, 30 isolates randomly selected from 2007-9 were included. These isolates were collected as part of the CANWARD study, and only included isolates collected from the same province, source, and age group as the other 162 isolates. Overall, 44 different serotypes were represented. Phylogenomic analysis was performed using in-house bioinformatics pipelines. Maximum likelihood trees were generated using PhyML, visualized using FigTree and cluster analysis was performed using ClusterPicker.

Results: A maximum likelihood tree generated against the NCBI reference strain SPN R6 (NC_003098) demonstrated that, in general, isolates clustered according to their serotype/serogroup. Isolates did not cluster by year or province of isolation. Examination of the clusters with multiple serotypes demonstrated a larger number of potential capsular switching events than previously recognized. Identification and examination of antimicrobial resistance determinants (β-lactams, macrolides, tetracyclines, fluoroquinolones and folic acid pathway inhibitors) indicates that MDR isolates in Canada have remained stable from 2007-14, and are present across the country.

Conclusion: The use of WGS has allowed increased insight into a highly diverse group of SPN isolates. Further analysis of these genomes is essential to understanding the evolution and population structure of SPN in Canada.

BACKGROUND

Streptococcus pneumoniae is a highly diverse organism capable of causing invasive disease in children, older adults and immunocompromised individuals [1]. Antimicrobial and multi-drug resistance (MDR) in *S. pneumoniae* is a growing concern, escalated by the worldwide dissemination of resistant and MDR international clones. Historically, a small number of the 90+ pneumococcal serotypes have accounted for the majority of invasive disease [1]. However, after using pneumococcal conjugate vaccines (PCV-7, PCV-13) for over a decade in Canada, serotype prevalence has shifted due to both replacement of vaccine types and vaccine escape through capsular switching events [2]. Previously, subtyping methods were used to help identify these switching events and genes associated with resistance. In recent years, whole genome sequencing (WGS) has become the method of choice to characterize isolates, due to its unambiguous examination of the total genetic content of a strain at the single nucleotide level [3].

The SAVE study is an annual study initiated in Canada in 2011, after PCV-13 introduction in Canada. Invasive *S. pneumoniae* isolated from sterile sites are forwarded from Canadian Public Health Laboratories to the Public Health Agency of Canada – National Microbiology Laboratory (PHAC-NML) for serotyping by the Quellung reaction. Through a collaboration between the Canadian Antimicrobial Resistance Alliance (CARA) and the PHAC-NML, these *S. pneumoniae* isolates were forwarded to CARA for further testing. A total of 5,012 isolates were sent to CARA from January 2011 to December 2014, inclusive.

The goal of this study was to utilize WGS to thoroughly characterize a subset of isolates collected by the SAVE study, to examine the distribution of MDR strains, as well as to identify capsular switching events.

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MATERIALS & METHODS

Antimicrobial Susceptibility Testing and Preliminary Molecular Characterization

Antimicrobial susceptibility testing was performed using custom-designed, in-house prepared broth microdilution panels, in accordance with Clinical and Laboratory Standards Institute (CLSI) guidelines [4]. MICs were interpreted using CLSI breakpoints [5], and MDR was defined as resistance to ≥3 antimicrobial classes (penicillin resistance MIC ≥2 μg/mL). Isolates resistant to ≥5 antimicrobial classes were defined as extensively drug-resistant (XDR). Ten isolates of each of the eleven most common serotypes per year (7F, 19A, 22F, 3, 12F, 11A, 9N, 8, 33F, 15A, 6C; 40 of each serotype, 440 total isolates) were characterized for genetic relatedness by pulsed-field gel electrophoresis (PFGE) and multi-locus sequence typing (MLST). PFGE was performed as previously described [6]; MLST was performed using methods and primers previously described at http://pubmlst.org/spneumoniae. Resulting PFGE fingerprints and MLST sequence types (STs) were compared to the Pneumococcal Molecular Epidemiology Network (PMEN) clone database. To assess putative virulence, PCR to determine the presence of pneumococcal pili was performed using previously described primers [7].

Isolate Selection for Whole Genome Sequencing

A total of 192 isolates were selected for WGS by the Illumina MiSeq platform. An initial 84 isolates from the SAVE study were specifically selected from the above serotypes due to preliminary characterization that indicated MDR, novel MLST STs and/or the potential to be a capsular switch variant. To achieve broader coverage of the diverse pneumococcal population, 78 additional isolates from SAVE were selected as "background". These isolates were selected using a random number generator, and included three of each PPV-23 vaccine serotype and up to three of other non-vaccine serotypes to total 78 isolates. To include isolates collected prior to PCV-13 introduction, 30 isolates randomly selected from 2007-9 were included. These isolates were collected as part of the CANWARD study; only isolates collected from the same provinces and source as the other 162 isolates were included. In an effort to control one of the many variables, all isolates were selected from the 65+ year age category, as this age group had the largest and most diverse collection of isolates from which to sample. Overall, 44 different serotypes were represented in this analysis.

Data Analysis

Phylogenomic analysis was performed using in-house bioinformatics pipelines. Maximum likelihood trees were generated using PhyML, visualized using FigTree and cluster analysis was performed using ClusterPicker. The presence of acquired resistance genes was determined using ResFinder 2.1, while genes with chromosomal mutations conferring resistance were extracted and compared to those of SPN R6 (NC_003098). Capsular switch variants were identified utilizing a previously described penicillin-binding protein (PBP) transpeptidase domain typing scheme [8].

CONCLUSIONS

1. Invasive *S. pneumoniae* in Canada are highly diverse. 192 isolates typed by WGS grouped into 35 clusters, of which 17 included isolates identical or related to PMEN clones.
2. A number of capsular switch events were identified, including those that aided in vaccine escape, creation of new MDR clones and diversification of otherwise uncommon serotypes.
3. MDR determinants were identified in a variety of serotypes over the course of the study period. Genotyping with WGS matched well to the corresponding phenotypic MIC results.
4. β-lactam resistance was most commonly associated with the presence of mutations in all three PBPs. Macrolide resistance was common, however the dual *mefA*+*ermB* genotype was only identified in serogroup 19 isolates related to PMEN14. Tetracycline and TMP-SMX resistance was identified in a wide variety of serotypes and conferred by well described resistance mechanisms *tetM* and *folA/P*, respectively. Fluoroquinolone and chloramphenicol resistance was minimal.

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RESULTS

Figure 1. Maximum likelihood tree of WGS data (192 isolates) generated using NCBI reference strain SPN R6 (NC_003098). Tree was generated using PhyML, visualized using FigTree and cluster analysis performed using ClusterPicker. Clusters with similarity to Pneumococcal Molecular Epidemiology Network (PMEN) clones are listed along with the representative serotype for that clone.

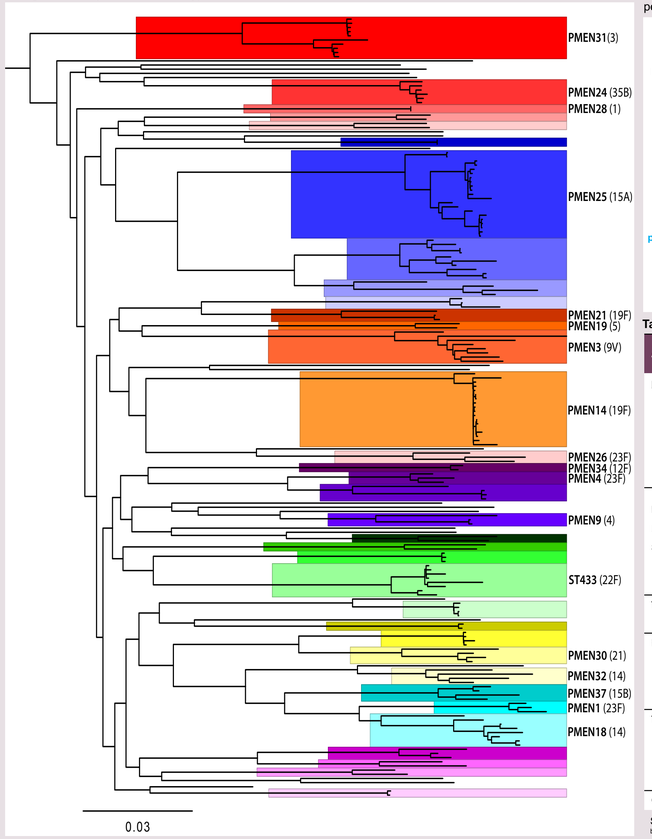

Figure 2. Visualization of an example of a capsular switch event. Identification was performed using a penicillin-binding protein transpeptidase domain typing scheme outlined by Metcalf et al [8]. Similarly to MLST, PBPs are assigned arbitrary allele numbers based on individual nucleotide differences from the penicillin-susceptible NCBI reference strain TIGR4 (NC_003028).

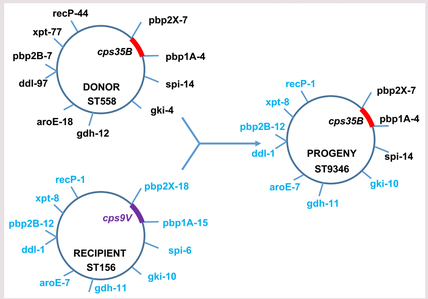

Table 1. Additional capsular switch events identified using the method outlined in Figure 2. Each switching event is important for a different reason: orange events transferred MDR to serotypes not normally MDR; yellow are vaccine escape events; blue events increased the diversity of generally uncommon serotypes.

Donor	Recipient	Progeny
8, 7F	PMEN25 (ST63-15A)	ST63-8, 7F
22F		ST9352-22F
19A		ST63-19A
24F, 10A	*PMEN 34 (ST230-14)	ST230-24F, 10A
19A		ST319-19A
31	22F (ST433)	ST433-31
*29	CLR-R 35B	CLR-R 29

*, the isolate was not part of the 192 WGS isolates, and is therefore a putative event at this time.

Table 2. Resistance genes identified in 192 *S. pneumoniae* isolates sequenced using WGS.

Antibiotic Class	Resistance Gene	Count (%)	S/I/R (n)	%S	%NS	Serotypes
β-Lactam	*pbp2B* only	19 (10.0)	5/14/0	26.3	73.7	6ABC(4), 7F(1), 8(1), 10A (1), 15A(6), 19A(2), 22F(2), 23B(2)
	pbp2X only	12 (6.3)	12/0/0	100	0	3(4), 5(1), 11A(1), 12F(1), 15B(1), 16F(1), 19A(2), 33F(1)
	1A+2B	1 (0.5)	0/1/0	0	100	24F(1)
	1A+2X	1 (0.5)	0/1/0	0	100	35B(1)
	2B+2X	7 (3.7)	2/4/1	28.6	71.4	6C(2), 15A(2), 19A(3)
	1A+2B+2X	42 (22.1)	0/10/32	0	100	6B(1), 9V(4), 15AB(7), 19AF(23), 23F(1), 29(1), 35B(5)
Macrolide/ Lincosamide/ Streptogramin	*mefA* only	20 (10.5)	1/2/17[a]	5.0	95.0	6ABC(6), 9V(2), 14(3), 12F(1), 15B(1), 19A(1), 22F(1), 29(1), 35B(4)
	ermB only	50 (26.3)	1/1/48[a]	2.0	98.0	3(6), 6BC(3), 7F (1), 8(1), 9N(1), 11A(2), 12F(2), 15AB(15), 17F(2), 19A(8), 22F(3), 23AF(2), 24F(1), 33F(3)
			9/0/41[b]	18.0	82.0	
	dual	18 (9.5)	1/2/15[a]	5.6	94.4	19A(17), 19F(1)
			7/0/11[b]	38.9	61.1	
Tetracycline	*tetM*	67 (35.3)	4/0/63	6.0	94.0	3(6), 6BC(3), 7F(1), 8(1), 9N(1), 10A(1), 11A(1), 12F(2), 15AB (15), 17F(2), 19AF(26), 22F(2), 23F(1), 24F(1), 25F(1), 33F(3)
Fluoroquinolone	*parC* S79 only	4 (2.1)	2/0/2[c]	50.0	50.0	11A(1), 19A(2), 22F(1)
	gyrA S81 only	3 (1.6)	2/1/0[d]	66.7	33.3	9N(1), 19A(1), 35B(1)
	both	8 (4.2)	0/0/8[c]	0	100	6A(2), 11A(1), 19A(1), 22F(2), 23F(2)
			0/4/4[d]	0	100	
TMP-SMX	*folA* I100L only	1 (0.5)	0/1/0	0	100	19A(1)
	folP mutation only	13 (6.8)	4/8/1	30.8	69.2	5(1), 10A(1), 15BC(4), 18C(1), 19A(1), 23B(1), 24F(1), 25F(1), 33F(2)
	both	38 (20.0)	0/1/37	0	100	5(1), 6ABC(4), 9V(4), 10A(1), 11A(2), 15AB(2), 19AF(21), 23F(1), 35B(2)
Chloramphenicol	*cat*	8 (5.0)	0/0/8	0	100	3(5), 15B(1), 19A(1), 23F(1)

S, susceptible; I, intermediate; R, resistant; NS, non-susceptible; TMP-SMX, trimethoprim-sulfamethoxazole. [a], susceptibility to clarithromycin. [b], susceptibility to clindamycin. [c], susceptibility to levofloxacin. [d], susceptibility to moxifloxacin.

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