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PROJECT TITLE: Evolution and Characterization of Penicillin-Resistant Streptococcus pneumoniae in Canadian Hospitals 2007-2010.

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SUMMARY: *Streptococcus pneumoniae* continues to cause considerable morbidity and mortality as a respiratory tract pathogen. Evolution and adaptation of *S. pneumoniae* occurs in response to antimicrobial use as well as vaccination of children and adults. The evolution of penicillin-resistant and multi-drug resistant (MDR) *S. pneumoniae* is of considerable concern to scientists and clinicians. In this study, penicillin-resistant isolates of *S. pneumoniae* were collected from the CANWARD national surveillance study over the years of 2007-2010 inclusive, and were studied to understand their demographic characterization, antimicrobial resistance patterns, serotype distribution, genetic and phenotypic relatedness, and virulence factors. This study determined penicillin-resistant *S. pneumoniae* from Canadian hospitals to be genetically related and frequently possess a MDR phenotype. These highly pathogenic pneumococci originated from all over Canada, and were isolated from patients from a wide age range in a variety of hospital settings. The emerging serotype 19A represented the most important source of penicillin and MDR strains. Clusters of penicillin-resistant *S. pneumoniae* isolates were genetically related to each other and to internationally recognized clones, including Taiwan^{19F}-14, Spain^{9V}-3, Spain^{23F}-1, and England^{14}-9. The pilus-encoding genetic islet virulence factors, PI-1 and PI-2 were associated with serotypes 19A, 19F, 9V, 14, and 35B, with PI-2 associated only with 19A and 19F isolates. In conclusion, penicillin-resistant *S. pneumoniae* accounted for 3.4% of all *S. pneumoniae* from Canadian hospitals between 2007-2010 and were isolated from patients of a wide age range, from all hospital ward types and all regions across Canada. These virulent *S. pneumoniae* were represented predominantly by the emerging serotype 19A, commonly exhibited a MDR phenotype, were genetically related and frequently possessed the virulence factors, P1 and P2.

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Introduction

Streptococcus pneumoniae (pneumococcus) is a major, globally distributed, increasingly concerning respiratory tract pathogen. Annually, pneumococcal disease is responsible for 1 million deaths among children <5 years of age[1]. *S. pneumoniae* is a leading bacterial cause of upper respiratory tract infections including otitis media and bacterial sinusitis, as well as invasive disease including community acquired pneumonia (CAP), bacteremia and meningitis[1-13]. Despite initially being extremely susceptible to penicillin[8,14], the rapid evolution of *S. pneumoniae* resistance to penicillin, as well as other β-lactams, macrolides, sulfonamides, tetracyclines and fluoroquinolones, is cause for concern [8-11,13,15-18]. Even more disturbing is that penicillin non-susceptible *S. pneumoniae* (PNSP, intermediate or resistant to penicillin) are more likely to concurrently exhibit resistance to other classes of antimicrobials[8,14], and thus frequently display a multidrug-resistant (MDR, resistant to ≥3 classes of antimicrobials) phenotype[7,8,11,12,14,17,18]. MDR is increasing at an alarming rate among *S. pneumoniae*[14], and 30% of *S. pneumoniae* worldwide are now MDR[6,14]. Rising antimicrobial resistance complicates treatment decisions, can cause treatment failures, and raises the costs of healthcare[11,19,20].

The principle virulence factor of *S. pneumoniae*, and the target for our current conjugate vaccines, is the polysaccharide capsule[19,21]. To date there are 91 distinct capsular serotypes known, organized into 46 distinct serogroups[2,14]. Significant differences in virulence, geographic distribution, tendency to colonize the nasopharynx, ability to cause invasive disease, case-fatality rates, and capacity to acquire drug resistance exist between different serotypes[2,19]. Genetic recombination through transformation is well known to occur in *S. pneumoniae*[22]. Co-colonizing strains of *S. pneumoniae* can exchange capsular genes through transformation as the genetic locus encoding the capsular polysaccharide (*cps*) is flanked by conserved sequences and is prone to recombination[22]. Thus, the pneumococcus is capable of switching its serotype which may lend an advantage in the face of vaccine pressure[21] and allow virulent strains to evade the vaccine, proliferate in the population, and acquire drug resistance[14,21]. Other virulence factors exist in *S. pneumoniae* as well. Recently, the discovery of pili in Gram-positive pathogens, including *S. pneumoniae*, has led to hypotheses regarding the role of pili in successful *S. pneumoniae* clones[23]. Two pili, called P1 and P2, have been discovered in *S. pneumoniae* [24,25]. The function of these pili in the virulence of the pneumococcus has been proposed to be related to mediating interactions between the bacterium and the host[24]. Namely, they have been noted to play a role as adhesins, aiding *S. pneumoniae* in adhering to respiratory epithelium[24,25], and there has been suggestion that P1 also acts as a pro-inflammatory stimulus[24]. The further study of P1 and P2 is warranted in order to better understand pneumococcal pathogenicity and virulence, and to explore these structures as future protein-based vaccine targets[25].

The introduction of the heptavalent pneumococcal vaccine (PCV7, Prevnar ®) in 2000 and its recommendation for all children <2 years old and children 2-4 with certain chronic conditions[20,26] has incited *S. pneumoniae* to undergo a major epidemiological shift. The vaccine has resulted in a substantial decrease in the overall occurrence of invasive disease [20-22,26-28]. Prior to the development of the vaccine, the seven pneumococcal serotypes included in PCV7 (4, 6B, 9V, 14, 18C, 19F, and 23F) were responsible for ~80% of invasive disease in children in the US[22,27]. Consequently, the vaccine has resulted in a tremendous reduction in disease caused by vaccine serotypes (VS) and vaccine-related serotypes (VRS, serotypes belonging to the same serogroup as a vaccine serotype)[22,26,27]. Worldwide, the serotypes most commonly associated

with antimicrobial resistance are 6B, 9V, 14, 19F, and 23F, all of which are included in PCV7[20,22]. Thus, vaccine use has also resulted in a reduction in illness caused by antimicrobial-resistant strains[20,22]. Unfortunately, the positive impact of the PCV7 vaccine has been marred by an increase in pneumococcal disease caused by non-vaccine serotypes (NVS), namely by the emerging serotype 19A[20-22,26,28]. The emergence and spread of serotype 19A is of particular concern as it has become the most significant cause of disease post-PCV7 and is frequently MDR [20,26,28,29].

Globally, the rise in antimicrobial resistance among *S. pneumoniae* is the consequence of several internationally disseminated clones[2,7]. Using pulsed-field gel electrophoresis (PFGE) and multi-locus sequence typing (MLST) methods, modern epidemiologic studies are concerned with characterizing the different clones or lineages within serotypes that share a common ancestor[30]. Selective pressure from PCV7 has influenced the serotypes and their clonal associations within the *S. pneumoniae* population. To better appreciate the complex evolutionary history of *S. pneumoniae* and to better understand and track the dissemination of successful clones, serotype and genotype relations must be further studied. This is especially important in Canada, where this type of research is currently lacking.

Hypotheses

i) Penicillin-resistant *S. pneumoniae* isolates from Canadian hospitals from 2007-2010 are frequently MDR.
ii) *S. pneumoniae* is a major pathogen across Canada, particularly among the young and old age groups.
iii) *S. pneumoniae* from Canadian hospitals are genetically related.
iv) *S. pneumoniae* in Canada is undergoing an epidemiological shift due to the influence of the heptavalent pneumococcal vaccine (PCV7, Prevnar ®).
v) The two pili, P1 and P2, associated with virulence of *S. pneumoniae* are commonly found in penicillin-resistant *S. pneumoniae* in Canadian hospitals.

Objectives

i) To assess the antimicrobial resistance patterns, including MDR, among penicillin-resistant *S. pneumoniae* isolates from Canadian hospitals from 2007-2010.
ii) To assess the genotypic and phenotypic relatedness of these isolates using PFGE.
iii) To demonstrate the influence of the heptavalent vaccine (PCV7, Prevnar ®) on the epidemiology of penicillin-resistant and MDR *S. pneumoniae*.
iv) To study the virulence of penicillin-resistant and MDR *S. pneumoniae* by assessing the genomic presence of pilus-encoding islets.

Materials and Methods

i) Bacterial Isolates

The purpose of the CANWARD study is to determine the nationwide prevalence of pathogens responsible for infections in Canadian hospitals and to characterize their antimicrobial resistance patterns. Annually, medical centers from across Canada were required to collect and submit clinically significant isolates (unique, consecutive; 1 organism per infection site per patient) from blood, respiratory, urine, and wound infections from patients in hospital clinics, emergency rooms, medical and surgical wards, and intensive care units (ICUs). These isolates were

transferred to the coordinating laboratory at the Heath Sciences Center, Winnipeg, Manitoba on Amies charcoal swabs, subcultured onto appropriate media, and stocked in skim milk at -80°C until MIC and other testing was performed. Of the 1615 *S. pneumoniae* isolates from the CANWARD national surveillance study, 55 [3.4%] penicillin-resistant strains and their associated patient demographics were obtained, and are the focus of this study.

ii) Antimicrobial Susceptibility Testing

After two subcultures from frozen stock, *in vitro* minimal inhibitory concentrations (MICs) were determined for amoxicillin-clavulanate, ceftriaxone, cefuroxime, clarithromycin, clindamycin, doxycycline, ertapenem, levofloxacin, linezolid, meropenem, moxifloxacin, penicillin, tigecycline, trimethoprim-sulfamethoxazole and vancomycin by the broth microdilution method, according to the Clinical and Laboratory Standards Institute (CLSI) guidelines[31,32]. The MICs were determined using custom-made 96-well microtiter panels containing doubling concentrations of antimicrobial agents. The antimicrobial agents used were obtained as laboratory-grade powders from their respective manufacturers, and stock solutions were made and dilutions performed as described by CLSI[31]. The microtiter panels were inoculated with organism to a final concentration of approximately 5 x 10^5 CFU/ml and incubated at 37^{o}C in room air before reading. MIC interpretive standards were according to CLSI breakpoints[32].

iii) Serotyping

Isolates were serotyped by the Quellung reaction method using sera from the Statens Serum Institut (Copenhagen, Denmark)[33].

iv) Assessment of Genetic Relatedness

The genetic relatedness of the *S. pneumoniae* isolates was determined by pulsed-field gel electrophoresis (PFGE) according to published methods[33,34]. Genomic DNA was digested with *Sma*I and then subjected to electrophoresis using a contour-clamped homogeneous electric field apparatus (CHEF DRIII, BioRad Laboratories, Hercules, CA.) at 9 ◦C for 18.5 h with switch times of 2–30 s. PFGE fingerprints were analyzed with BioNumerics v3.5 (Applied Maths Inc., Austin, TX) and a dendrogram was generated by the unweighted pair group method with arithmetic averages[33] to demonstrate the genetic relatedness of the isolate cohort. The PFGE fingerprints were also compared to the Pneumococcal Molecular Epidemiology Network (PMEN) clonal database.

v) Assessment of Virulence

The virulence of the isolates was studied by detecting the presence of PI-1 and PI-2 adhesion pilus-encoding islets using PCR, as previously described[35]. DNA was extracted from the isolates using the Roche High Pure PCR Template Preparation Kit and used as a template for PCR. A different PCR reaction with its own unique set of primers was used to test each for the presence and absence of both PI-1 and PI-2 for each isolate. Thus, four individual PCR reactions were carried out per isolate.

- *PCR Detection of* PI-1*:*

The presence of PI-1 was determined by amplifying the *srtC/D* (sortase) gene which is present within the PI-1 pathogenicity islet. The *srtC/D* gene was amplified using the primers: Rlr_up_F, 5'-CTTCCACGAAGTTCTTTCAATGG-3' and Rlr_do_R, 5'-

GTCTTAGAATATCATGGTTTACGTGC-3' (PCR product, 600bp)[35]. The absence of PI-1 was tested using primers complimentary to the flanking genes surrounding PI-1. These primers were: Rlr_srtC_F, 5'-GGGGAAGATTATGCGACCTT-3' and Rlr_srtD_R, 5'-GCTTGGCTCTGCACGGTGCC-3' (PCR product, 700 bp)[35]. PI-1 is a 14 kb mobile genetic element[35] that, when present, is too large to yield a PCR product. Therefore, amplification of the sequence between these primers is consistent with the absence of PI-1. PCR products were analyzed by gel electrophoresis at 100 Volts for 30 minutes through 2% agarose gels to which ethidium bromide had been added. The gels were visualized using an AlphaImager® gel documentation system (Alpha Innotech Corporation, San Leandron, CA).

- *PCR Detection of* PI-2*:*

The presence of PI-2 was detected by amplifying the *sipA* gene which is present within PI-2, as previously described[35]. The primers used were as follows: sipA_up_F, 5'-CTCTAGGAGGGATCTTCTTTATCATC-3' and sipA_do_R, 5'-CTACAGCCGTGTTCGATTGTCC-3' (PCR product, 500 bp)[35].

Results

i) Patient Demographics

Of the 1615 *S. pneumoniae* isolated from 2007-2010 from Canadian hospitals, 55 (3.4%) were found to be penicillin-resistant (MIC ≥2ug/ml). These 55 penicillin-resistant *S. pneumoniae* are the focus of this investigation. The demographic data for the patients from which these 55 isolates were obtained can be viewed in Table 1. The average age of the patients was 46.4 with an age range of 1-89 (data not shown). Of the 55 isolates, 34 (61.8%) were from male patients while 21 (38.2%) came from female patients. Most isolates (41, 74.5%) came from respiratory tract specimens, while 14 (25.5%) were isolated from blood. The majority of isolates (25, 45.5%) came from the Prairie region of Canada (Manitoba and Saskatchewan) followed closely by Ontario and Quebec with 22 isolates (40.0%). Western (BC and Alberta) and Eastern (Nova Scotia and New Brunswick) Canada were each the source of only 4 isolates (7.3% each). The 18-64 age group demonstrated the greatest proportion of isolates (47.3%), followed by the >65 age group (29.1%), and the <17 group (23.6%). Isolates were obtained from all ward types, with the greatest percentage from emergency rooms (32.7%) and general medicine (23.6%). Isolates from the Clinic and ICU settings each represented 18.2% of the total, while the smallest proportion of isolates (7.3%) were from general surgery.

ii) Antimicrobial Susceptibility

The summarized results of susceptibility testing for select antimicrobials can be seen in Table 2. All isolates in the cohort demonstrated resistance to penicillin (MIC ≥2µg/ml). Very high level penicillin resistance (MIC ≥4 µg/ml) was observed in 23 (41.8%) isolates (data not shown). Beta-lactam resistance rates were 36.4% for amoxicillin/clavulanate (MIC ≥8 µg/ml), 96.4% for cefuroxime (MIC ≥4 µg/ml), and 1.8% for ceftriaxone (MIC ≥4 µg/ml). The majority of isolates (89.1%) were resistant to trimethoprim/sulfamethoxazole while 69.1% of isolates were resistant to clarithromycin, and 49.1% displayed resistance to clindamycin. Also, 98.2% of isolates tested were susceptible to moxifloxacin and all isolates were susceptible to vancomycin.

An analysis of the MDR phenotype, broken down according to the number of antimicrobial

classes to which the isolates displayed resistance, can be viewed in Table 3. It can be seen that the majority of isolates (67.3%) in the cohort were MDR. Additionally, a significant proportion (49.1%) were MDR displaying resistance to four or more classes of antimicrobials, and 2 isolates (3.6%) were MDR with resistance to five antimicrobial classes.

iii) Serotyping

The serotype distribution was: 19A (38.2%), 9V (20.0%), 19F (14.5%), 23F (10.9%), 14 (7.3%) and 35B (5.5%) [data not shown]. Together, these six serotypes represented 53 (96.4%) of 55 isolates. The remaining two isolates (3.6%) were non-typeable. Thus, serotype 19A was the most common serotype, representing 38.2% of isolates overall. Of the 37 MDR isolates resistant to three or more antimicrobial classes, 21 (56.8%) were serotype 19A, 6 (16.2%) were 19F, 4 (10.8%) were 23F, 3 (8.1%) were 9V, 2 (5.4%) were 14 and 1 (2.7%) was NT (Table 4). Of the 27 MDR isolates resistant to four or more antimicrobial classes, 19 (70.4%) were 19A, 6 (22.2%) were 19F, 1 (3.7%) was 9V, and 1 (3.7%) was 23F. Both isolates (100%) exhibiting resistance to five or more antimicrobial classes were serotype 19A.

iv) Genetic Relatedness

PFGE and dendrogram analysis revealed five major clusters accounting for 54 of the 55 (98.2%) isolates (Figure 1). The characterization of these clusters can be viewed in Table 5. The largest cluster (#1) consisted of 28 (50.9%) organisms, with 19F and 19A serotypes accounting for 8 (28.6%) and 20 (71.4%) of the isolates, respectively. In this cluster, 26 isolates (92.9%) were MDR, and resistance rates were 89.3% to clindamycin, 96.4% to clarithromycin, and 92.9% to trimethoprim-sulfamethoxazole. This cluster had the highest rates of MDR and resistance to clindamycin and clarithromycin, and contained all of the 19F and all but one of the emerging 19A serotypes. Twenty-five (96.2%) of the 26 MDR isolates belonging to cluster #1 were resistant to 4 or 5 distinct classes of antimicrobials. Interestingly, all of the high level penicillin resistant (MIC $\geq$4 μg/ml) isolates in the cohort were also found in cluster #1. Thus, cluster #1 mostly contained highly penicillin-resistant and MDR serotype 19A isolates.

The second largest cluster (#2) was composed of 14 of the 55 isolates (25.5%), with 11 (78.6%) serotype 9V, 2 (14.3%) serotype 14, and 1 (7.1%) serotype 19A organisms. In this cluster, 5 isolates (35.7%) were MDR, and resistance rates were 14.3% to clindamycin, 35.7% to clarithromycin, and 92.6% to trimethoprim-sulfamethoxazole. It is interesting to note that one of the 19A isolates was more closely related to the serotype 9V and 14 isolates in cluster #2 than all the other 19A and 19F isolates in cluster #1.

The third largest cluster (#3) included 6 of the 55 isolates in the cohort (10.9%) and consisted entirely of serotype 23F organisms. Within this cluster, 4 isolates (66.7%) were MDR, and resistance rates were 0.0% to clindamycin, 66.7% to clarithromycin, and 100% to trimethoprim-sulfamethoxazole. Clusters 4 and 5 were small, both containing only 3 isolates (5.5% each). Cluster 4 contained 3 isolates of serotype 35B, none of which were MDR. Resistance rates in this cluster were quite low at 0.0% to clindamycin, 0.0% to clarithromycin, and 33.3% to trimethoprim-sulfamethoxazole. Cluster 5 was made up of 2 serotype 14 isolates and a single non-typeable (NT) isolate. In this cluster, 2 isolates (66.7%) were MDR, and resistance rates were 0.0% to clindamycin, 66.7% to clarithromycin, and 100% to trimethoprim-sulfamethoxazole. Finally, one non-typeable isolate was only distantly related to the other

organisms in the cohort. It was not MDR and lacked resistance to clindamycin, clarithromycin, and trimethoprim-sulfamethoxazole.

v) Assessment of Virulence

The results of PCR testing for the presence of P1 and P2 pilus encoding islets can be seen in Figure 1. All of the isolates (except one) within the largest cluster (#1) possessed the *srtC*/*D* (sortase) gene indicating the presence of P1, and all isolates possessed the *sipA* gene indicating the presence of P2. Conversely, none of the isolates within clusters #3 and #5 possessed either the *srtC/*D or *sipA* gene, suggesting the absence of both P1 and P2. All but one of the isolates in cluster #2 demonstrated the presence of the *srtC/D* gene, confirming the presence of only P1 in these isolates. The one exception in cluster #2 was the 19A isolate which did not possess either *srtC/D* or *sipA* genes, and thus did not express either P1 or P2. The isolates in cluster #4 were found to only have *srtC/D*, and therefore possessed P1 only. Finally, the stand alone non-typeable isolate lacked both genes and hence had neither pilus. Overall, 43 of the 55 isolates (78.2%) possessed P1, and 28 (50.9%) possessed P2.

Discussion

Streptococcus pneumoniae persists as a major pathogen resulting in significant morbidity and mortality in Canada and worldwide[8]. Classically, *S. pneumoniae* has been particularly devastating to children (≤2 years) and older adults (≥65 years), with the highest incidence of invasive disease occurring in these age groups[7,19]. The data from this report, however, remind us that the most worrisome *S. pneumoniae*, that is penicillin-resistant *S. pneumoniae*, is an important pathogen in all age groups, including adults 18-64 years old. Penicillin-resistant *S. pneumoniae* isolates from CANWARD 2007-2010 represented ~3.4% of all *S. pneumoniae* isolates, came from 74.5% respiratory and 25.5% blood specimens from both male and female patients (with a slight male preponderance), affected an age range of 1-89 years, and was present on all ward types, most commonly emergency wards. Isolates originated from across the nation but were especially prevalent in the Prairie provinces (Manitoba, Saskatchewan), Ontario and Quebec. Thus, penicillin-resistant *S. pneumoniae* is a widely distributed, national concern in Canada, especially due to the increasing prevalence of MDR[7,14].

Among the penicillin-resistant *S.pneumoniae* from CANWARD 2007-2010, high percentages of resistance to macrolides (clarithromycin), lincosamides (clindamycin), trimethoprim-sulfamethoxazole, and cefuroxime were observed, and over a third of isolates were resistant to amoxicillin-clavulanate. It was found that the prevalence of MDR among the penicillin-resistant isolates was very high at 67.3%. This reflects the observation that organisms resistant to penicillin tend also to be resistant to other classes of antimicrobials, as has been reported in other studies[8,11,14]. The rates of MDR *S. pneumoniae* are escalating in Canada and globally, which presents a challenge to the treatment of pneumococcal disease. The relationship between drug resistance and treatment failures for *S. pneumoniae* infection is often complicated by other factors (such as patient age, comorbid illness, immunosuppression, initial antimicrobial choice, infection severity, and others)[2,4]. Nevertheless, the fact that initial treatment of community acquired pneumonia (CAP) is often empirical necessitates the knowledge of local antimicrobial susceptibility patterns for efficacious treatment[14]. Additionally, treatment failures for numerous β-lactam agents (including cephalosporins such as cefuroxime), macrolides, trimethoprim-sulfamethoxazole, and tetracyclines have been reported[11,18]. According to the Canadian

Infectious Disease Society/Canadian Thoracic society guidelines on the initial management of CAP, either a respiratory fluoroquinolone such as moxifloxacin (98.2% susceptible in our study) or combination therapy with a macrolide and β-lactam agent are preferred empiric treatments. The susceptibility results from our data support these empiric treatment options. However, escalating antimicrobial resistance to macrolides and β-lactams among *S. pneumoniae* has potential to complicate antimicrobial therapy and limit the number of available treatment options. With respect to inpatients, ICU patients, patients with comorbid illness, and treatment of penicillin-resistant (MIC ≥2μg/ml) *S. pneumoniae,* respiratory fluoroquinolones (such as moxifloxacin or levofloxacin) are recommended therapy. Again, our data support respiratory fluoroquinolone treatment in these treatment settings, although recent reports of rising numbers of fluoroquinolone-resistant pneumococci are concerning[11,14,18]. The judicious use of fluoroquinolones and further antimicrobial susceptibility monitoring studies need to be undertaken in order to preserve and protect the activity of fluoroquinolones against *S. pneumoniae*. Treatment options for known penicillin-resistant strains are quite limited and include respiratory fluoroquinolones, vancomycin, linezolid, and high dose penicillin.

The data from this study are a prime example of the epidemiological impact of the PCV7 vaccine on the *S. pneumoniae* population. Several authors have proposed that the post-vaccine success favoring 19A was a combination of several factors[29]. For instance, aside from the PCV7 included serotypes and the vaccine-related serotype 6A, 19A was the most common cause of IPD before the introduction of PCV7. Also, 19A is equivalently able to colonize the nasopharynx, and cause otitis media and invasive disease. It frequently demonstrates penicillin and multidrug-resistance, providing it a selective advantage, and PCV7 does not provide cross-reactive protection against serotype 19A[29]. These factors together set the stage for the replacement of vaccine-serotypes with 19A. Since the year 2000, serotype 19A has risen to become the most common cause of invasive disease and is most rapidly acquiring MDR[14,19,26,29]. These disturbing observations are evident in our data, as serotype 19A was the predominant serotype (38.2% of the cohort) and had the highest rates of MDR (all 20 serotype 19A isolates were MDR). Additionally, the MDR serotype 19A isolates in the cohort consistently exhibited resistance to 4 or more distinct classes of antimicrobials, with 17 isolates resistant to 4 antimicrobial classes and 2 isolates being resistant to 5 classes of antimicrobials. The increasing propensity of serotype 19A to acquire resistance makes this serotype a growing problem and potentially difficult to treat.

Pneumococcal clonal dynamics in the post-vaccine era are well demonstrated in our data. Clusters related to Taiwan^{19F}-14, Spain^{23F}-1, Spain^{9V}-3, and England^{14}-9 Pneumococcal Molecular Epidemiology Network (PMEN) clones were observed, as have been previously reported [36,37]. This suggests clonal spread as the mechanism underlying increasing drug resistance in Canada. The largest cluster identified by PFGE, consisting entirely of serotype 19F and 19A organisms, was most closely related to Taiwan^{19F}-14, a MDR internationally established pre-PCV7 clone. The Taiwan^{19F}-14 clone (sequence type [ST] 236 by MLST) is closely related to ST320 and ST271 clones, and all are classified in clonal complex 271 (CC271). ST320 and ST271 are associated with both serotypes 19A and 19F[28,29], and have played a large role in the increase in MDR serotype 19A in the post-vaccine era[38,39]. Evidence for capsular switching from 19F to 19A has been found for each of the Taiwan^{19F}-14, ST320, and ST271 clones[28,37]. Further studies need to be undertaken to understand the virulence factors and selective advantages contributing to the success of these clones, in addition to vaccine escape. In future work,

examining the prevalence of ST320/271 using MLST would delve further into the clonal changes ongoing within serotype 19A. The second largest pulsed-field cluster was composed of serotype 9V and 14 isolates, and was most closely related to the Spain^{9V}-3 clone (ST156). The ST156 identifier of the Spain^{9V}-3 clone is primarily associated with serotypes 9V and 14[26]. Interestingly, capsular switching events have recently been documented identifying the presence of ST156 within serotype 19A[26]. Thus, the presence of the 19A isolate found in this study that is genetically grouped with the serotype 9V and 14 isolates may be hypothesized to be representative of a capsular switching event between a 19A donor and a 9V or 14 ST156 recipient. Given the observed increase in the drug resistant ST156 clone post-PCV7[25], MLST could be an important next step to study this hypothesis in future work. Finally, another interesting PFGE finding is the peculiar grouping of a non-typeable (NT) isolate with two other serotype 14 isolates, most closely related to the England^{14}-9 clone. A recent report from Brazil has confirmed the close genetic relatedness of NT isolates and serotype 14, and has suggested that NT isolates resulting from capsular switching may serve as a reservoir for replacement disease post-PCV7[40]. This study also demonstrated that NT isolates frequently lack capsular genes and thus either do not produce, or produce a very small capsule undetectable with the Quellung reaction method. Future work could employ PCR to study the capsular gene status of the NT isolates in our cohort.

The role of two pili, P1 and P2, in pneumococcal virulence is emerging as a subject of interest. Prior to PCV7 use, pilus-type 1 was present in approximately 25% of isolates and was associated primarily with vaccine-type strains[24]. It followed then, that the dramatic decline in vaccine-serotypes after the introduction of the vaccine was mirrored also by a sharp decline in the prevalence of P1. Since then, P1 has undergone a resurgence and is now more prevalent than before PCV7, and has been noted to be increasing in frequency among replacement non-vaccine serotypes[24]. This study found P1 to be present in penicillin-resistant *S. pneumoniae* of serotypes 19A, 19F, 9V, 14, and 35B. Thus, the association of P1 with emerging non-vaccine serotypes (including 19A and 35B) is demonstrated in this study. The presence of P1 in vaccine serotypes 19F, 9V, and 14 is interesting. Despite a reduction in vaccine serotypes and P1 prevalence due to PCV7, this study indicates that P1 remains highly associated with certain vaccine serotypes. Additionally, the presence of P1 has been noted to be a clonal property, and has been associated with the Spain^{9V}-3 (ST156)[7,23] and Taiwan^{19F}-14 (ST236)[7] clones, and also with the emerging serotype 35B ST558 clone[23]. This is demonstrated nicely in this study, as isolates in the clusters related to the Taiwan^{19F}-14 and Spain^{9V}-3 clones possessed P1. It would be interesting to employ MLST in the future to determine whether the P1 piliated serotype 35B isolates were related to the ST558 clone. Among penicillin-resistant *S. pneumoniae*, both the continued association of P1 with certain vaccine serotypes and the emergence of P1 among non-vaccine serotypes are evident in this study. A correlation between P1 presence and penicillin-resistance has been previously identified[24], and the interaction between these two virulence factors in aiding the success of these emerging pathogens warrants further study. Pilus-type 2, however, was exclusively present among all the 19A and 19F isolates in the pulsed-field cluster related to Taiwan^{19F}-14, and all but one of these isolates possessed both P1 and P2. Consistent with this data, P2 has been previously associated with serotypes 1, 2, 7F, 19F, and 19A[25]. Interestingly, the presence of P1 and P2 together has only been noted for CC271, including the Taiwan^{19F}-14 clone[25]. Thus, investigating the association between clonal type and pilus presence within this

cluster using MLST would further delineate the relationship between clonal success and pilus-related virulence.

SIGNIFICANT FINDINGS/CONCLUSIONS

1. Penicillin-resistant *S. pneumoniae* from Canadian hospitals are frequently MDR.
2. Penicillin-resistant and MDR *S. pneumoniae* affect all ages of patients, and are found in all regions across Canada and in all hospital ward types, especially emergency rooms.
3. Penicillin-resistant *S. pneumoniae* in Canada are genetically related. Pulsed-field gel electrophoresis analysis revealed clusters of isolates related to Taiwan^{19F}-14, Spain^{9V}-3, Spain^{23F}- 1, and England^{14}-9 clones. This suggests clonal expansion as a mechanism underlying penicillin-resistance and MDR dissemination in Canada.
4. The impact of the PCV7 vaccine is clearly evident in the *S. pneumoniae* population in Canada. The increase in non-vaccine serotype 19A and changing genetic relationships are a consequence of vaccine selection pressure.
5. PI-1 and PI-2 pilus-encoding islets are frequently present among penicillin-resistant and MDR *S. pneumoniae*, and likely provide these organisms increased virulence.

CLINICAL RELEVANCE

The empiric treatment of community-acquired respiratory infections such as CAP is complicated by the increasing prevalence of MDR *S. pneumoniae* in Canada. Drug resistance has the potential to incite confusion around therapeutic decisions, cause treatment failures, and increase the cost of healthcare. Despite the positive impact of the PCV7 vaccine, invasive disease caused by replacement serotypes is a growing problem. Future polysaccharide vaccines, such as the new PCV13 vaccine, must target these replacement serotypes, especially 19A. Furthermore, new serotype-unrelated vaccine targets, such as pilus antigens, have potential to avoid some of the pitfalls of polysaccharide vaccines and deserve future research attention. Finally, future epidemiologic surveillance studies are warranted in order to better appreciate the current genetic changes ongoing in the pneumococcal population and to understand further what are important factors contributing to clonal success.

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Table 1. Demographic characterization of patients from which penicillin-resistant *S. pneumoniae* (n=55) was isolated.

Demographic Characteristic	Blood (n=14)	Percentage of Isolates (n=14)	Respiratory (n=41)	Percentage of Isolates (n=41)	Row Total	Percentage of Isolates (n=55)
i) Sex						
Male	10	71.4	24	58.5	**34**	**61.8**
Female	4	28.6	17	41.5	**21**	**38.2**
ii) Age Group						
≤17 years	7	50.0	6	14.6	**13**	**23.6**
18-64 years	4	28.6	22	53.7	**26**	**47.3**
≥65 years	3	21.4	13	31.7	**16**	**29.1**
iii) Region of Canada						
Western (British Columbia, Alberta)	2	14.3	2	4.9	**4**	**7.3**
Prairie (Manitoba, Saskatchewan)	8	57.1	17	41.5	**25**	**45.5**
Ontario/Quebec	4	28.6	18	43.9	**22**	**40.0**
Eastern (Nova Scotia, New Brunswick)	0	0	4	9.8	**4**	**7.3**
iv) Hospital Ward Category						
General Unspecified ICU	3	21.4	7	17.1	**10**	**18.2**
Emergency Room	9	64.3	9	22.0	**18**	**32.7**
Clinic/Office	0	0	10	24.4	**10**	**18.2**
Medicine General	2	14.3	11	26.8	**13**	**23.6**
Surgery General	0	0	4	9.8	**4**	**7.3**

Table 2. Antimicrobial susceptibility of 55 penicillin-resistant *S. pneumoniae* isolates.

Antibiotic	MIC_{50} (µg/ml)	MIC_{90} (µg/ml)	MIC Range (µg/ml)	% of Isolates per Category[a]		
				S	I	R
Penicillin (PEN)	2	4	2 – 8	0	0	100
Amoxicillin/ Clavulanate (A/C)	4	8	0.5 - 8	45.5	18.2	36.4
Clarithromycin (CLR)	>32	>32	</=0.03 - >32	27.3	3.6	69.1
Clindamycin (CD)	0.5	>64	</=0.12 - >64	49.1	1.8	49.1
SXT[b]	8	>8	</=0.12 - >8	7.3	3.6	89.1
Doxycycline (DOX)	4	4	</=0.25 – 8	N/A[c]	N/A[c]	N/A[c]
Moxifloxacin (MXF)	0.12	0.25	0.12 – 2	98.2	1.8	0
Cefuroxime (CXM)	4	16	1 – 16	1.8	1.8	96.4
Ceftriaxone (CRO)	1	2	</=0.12 – 4	87.3	10.9	1.8
Vancomycin (VAN)	0.25	0.5	</=0.12 – 0.5	100	0	0

[a]S, susceptible; I, intermediate; R, resistant. [b]SXT, Trimethoprim-sulfamethoxazole
[c]There are no CLSI breakpoints presently defined for doxycycline.

Table 3. Prevalence of multi-drug resistance (MDR) among 55 penicillin-resistant *S. pneumoniae* isolates.

MDR[a] Category	No. of Isolates	% of isolates (n=55)
≥3 Classes	37	67.3
≥4 Classes	27	49.1
≥5 Classes	2	3.6

[a]MDR, multi-drug resistant. Defined as resistance to at least one antimicrobial agent from three or more different classes.

Table 4. Serotype breakdown of MDR *S. pneumoniae* isolates.

No. of antimicrobial classes	**No. (% of row total) of each serotype**							**Row Total**	**% of cohort (n=55)**
	19A	19F	14	9V	23F	35B	NT		
≥3	**21 (56.8)**	6 (16.2)	2 (5.4)	3 (8.1)	4 (10.8)	0 (0.0)	1 (2.7)	**37**	**67.3**
≥4	**19 (70.4)**	6 (22.2)	0 (0)	1 (3.7)	1 (3.7)	0 (0.0)	0 (0.0)	**27**	**49.1**
≥5	**2 (100)**	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	**2**	**3.6**

Table 5. Characterization of PFGE clusters of 55 penicillin-resistant *S. pneumoniae* isolates.

PFGE Cluster	Related PMEN clone within cluster	No. (% of total cohort) of isolates in cluster	No. (% of cluster) of MDR isolates	Serotypes (No. of isolates) within cluster
#1	**Taiwan^{19F}-14**	**28 (50.9)**	**26 (92.9)**	**19A (20), 19F (8)**
#2	Spain^{9V}-3	14 (25.5)	5 (35.7)	9V (11), 14 (2), 19A (1)
#3	Spain^{23F}-1	6 (10.9)	4 (66.7)	23F (6)
#4	N/A[a]	3 (5.5)	0 (0.0)	35B (3)
#5	England^{14}-9	3 (5.5)	2 (66.7)	14 (2), NT[b] (1)
Unique[c]	N/A[a]	1 (1.8)	0 (0.0)	NT[b] (1)

[a]This cluster/isolate was not found to be closely related to a particular PMEN clone.
[b]NT-Non-typeable.
[c]-This isolate was not part of a cluster.

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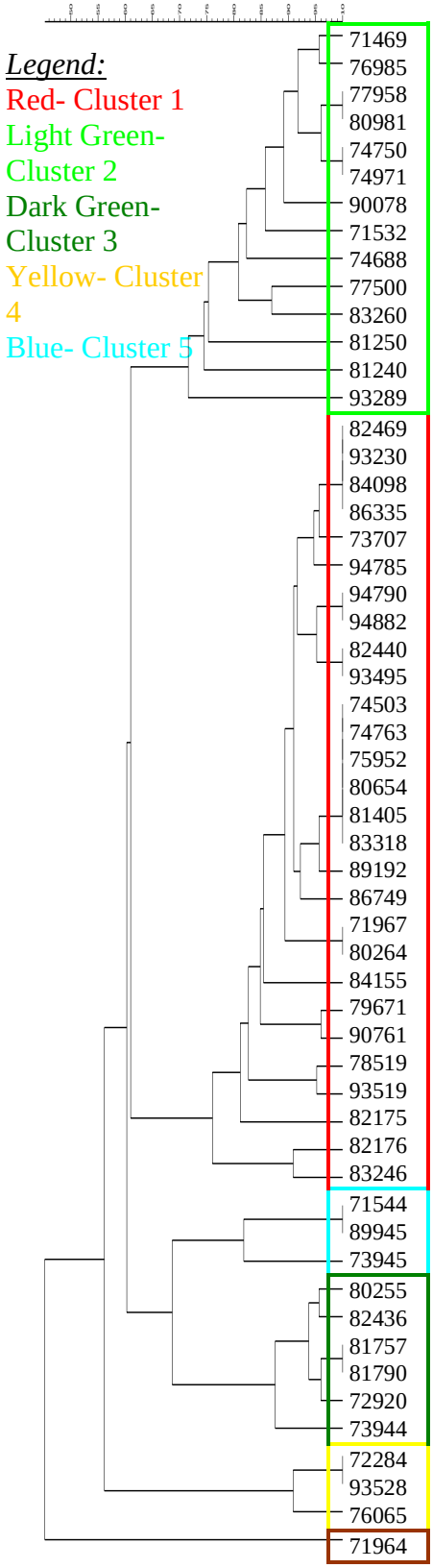
Figure 1. Genetic relatedness of 55 *S. pneumoniae* in Canadian hospitals from 2007-2010.



	Serotype	Pen MIC	P1 Present	P2 Present	MDR (# Classes)
71469	9V	2	+	-	No
76985	9V	2	+	-	No
77958	9V	2	+	-	No
80981	9V	2	+	-	No
74750	9V	2	+	-	No
74971	9V	2	+	-	No
90078	9V	2	+	-	Yes (3)
71532	14	2	+	-	No
74688	9V	2	+	-	Yes (3)
77500	9V	2	+	-	No
83260	9V	2	+	-	No
81250	14	2	+	-	Yes (3)
81240	9V	2	+	-	Yes (4)
93289	19A	4	-	-	Yes (3)
82469	19F	4	+	+	Yes (4)
93230	19F	4	+	+	Yes (4)
84098	19F	4	+	+	Yes (4)
86335	19F	4	+	+	Yes (4)
73707	19A	2	+	+	Yes (4)
94785	19A	4	+	+	Yes (4)
94790	19A	8	+	+	Yes (5)
94882	19A	4	-	+	Yes (4)
82440	19F	4	+	+	Yes (4)
93495	19A	4	+	+	Yes (4)
74503	19A	2	+	+	Yes (5)
74763	19A	2	+	+	Yes (4)
75952	19A	4	+	+	Yes (4)
80654	19A	4	+	+	Yes (4)
81405	19A	4	+	+	Yes (4)
83318	19A	4	+	+	Yes (4)
89192	19A	4	+	+	Yes (4)
86749	19A	4	+	+	Yes (3)
71967	19A	2	+	+	Yes (4)
80264	19A	4	+	+	Yes (4)
84155	19A	4	+	+	Yes (4)
79671	19A	4	+	+	Yes (4)
90761	19A	4	+	+	Yes (4)
78519	19F	4	+	+	Yes (4)
93519	19A	4	+	+	Yes (4)
82175	19A	4	+	+	Yes (4)
82176	19F	2	+	+	No
83246	19F	2	+	+	No
71544	NT	2	-	-	Yes (3)
89945	14	2	-	-	Yes (3)
73945	14	2	-	-	No
80255	23F	2	-	-	Yes (3)
82436	23F	2	-	-	No
81757	23F	2	-	-	Yes (3)
81790	23F	2	-	-	Yes (3)
72920	23F	2	-	-	No
73944	23F	2	-	-	Yes (4)
72284	35B	2	+	-	No
93528	35B	2	+	-	No
76065	35B	2	+	-	No
71964	NT	2	-	-	No