Evolution and molecular characterization of macrolide-resistant Streptococcus pneumoniae in Canada between 1998 and 2008

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†See the Acknowledgements section.

Received 15 April 2013; returned 13 May 2013; revised 19 July 2013; accepted 23 July 2013

Objectives: Studies were performed to assess resistance mechanisms, multidrug resistance (MDR), genetic relatedness, serotype distribution, heptavalent pneumococcal conjugate vaccine (PCV7) coverage and pili virulence factors among macrolide-resistant Streptococcus pneumoniae (MRSP) isolated from respiratory samples submitted to hospital laboratories across Canada from 1998 to 2008.

Methods: Isolates of MRSP (n = 1518) collected by the national surveillance studies CROSS (Canadian Respiratory Organism Susceptibility Study; 1998–2006) and CANWARD (Canadian Ward Surveillance Study; 2007–08) were tested using the CLSI broth microdilution method to establish antimicrobial susceptibilities. PCR was used to detect macrolide resistance genes [mef(A) and erm(B)] and pili virulence factors (type 1 pili and type 2 pili), the Quellung reaction was used to identify serotypes and PFGE was used to determine genetic relatedness.

Results: The prevalence of MRSP increased from 8% in 1998 to 22% in 2008 (P = 0.0001). MRSP were 51% mef(A) positive, 36% erm(B) positive, 8% dual mef(A) and erm(B) positive and 5% mef(A) and erm(B) negative. Dual mef(A)- and erm(B)-positive isolates increased in prevalence from 3% in 1998 to 19% in 2008 (P = 0.001). The prevalence of PCV7 serotypes (4, 6B, 9V, 14, 18C, 19F and 23F) decreased from 67% in 1998 to 31% in 2008 (P = 0.0072). The prevalence of serotype 19A, a non-PCV7 serotype, increased by 15% from 1998 to 2008; isolates of serotype 19A were MDR, dual mef(A) and erm(B) positive, genetically related by PFGE and associated with the presence of pili virulence factors.

Conclusions: From 1998 to 2008, respiratory isolates of MRSP in Canada increased significantly due primarily to the emergence and spread of serotypes 6A, 19A and other non-PCV7 serotypes.

Keywords: surveillance, pili, vaccines, serotypes

Introduction

Streptococcus pneumoniae is a major cause of morbidity and mortality worldwide, especially among children and the elderly.1 It is a major cause of community-acquired respiratory tract infections (CARTIs) such as community-acquired pneumonia (CAP), acute otitis media and acute sinusitis, as well as life-threatening invasive pneumococcal diseases (IPDs) such as bacteraemia, septicaemia and meningitis.1 Macrolides are first-line agents recommended for the empirical treatment of CARTIs, particularly in cases of CAP, for amoxicillin failure and in cases of resistance or allergy to agents of other structural classes.2 The clinical management of these infections has become increasingly complicated by the worldwide emergence and spread of macrolide resistance and multidrug resistance (MDR) in S. pneumoniae.3,4

There are two primary mechanisms of macrolide resistance in S. pneumoniae: methylation of target site 23S RNA conferred by the erm(B) gene and efflux of macrolides from the cell through expression of the mef(A) gene.5 The erm(B) gene reduces the ribosomal binding affinity of 14-, 15- and 16-membered macrolides, lincosamides and streptogramin B antimicrobials (MLSb phenotype) and is associated with high-level macrolide resistance (MIC >64 mg/L). There is no description of the mef(A) gene in S. pneumoniae, but there is evidence that it is also associated with low-level macrolide resistance in S. pneumoniae (MIC 1-32 mg/L).5
A recent trend in macrolide resistance is the emergence of clinical isolates harbouring both erm(B) and mef(A) genes, referred to as the ‘dual’ phenotype. The dual phenotype has been increasingly described in macrolide-resistant S. pneumoniae (MRSP) worldwide. These isolates are often MDR, highly resistant to MLSB antimicrobials, penicillin, tetracycline, chloramphenicol and trimethoprim/sulfamethoxazole, genetically related and spread by clonal dissemination.

The capsule is the principal virulence factor in S. pneumoniae and the target for serotype-specific prevention of disease by vaccination. The heptavalent pneumococcal conjugate vaccine (PCV7) containing the seven most common serotypes causing IPD in infants and children, has significantly reduced the incidence of childhood IPD. It has also indirectly reduced antimicrobial resistance because many of the PCV7 serotypes harboured antimicrobial resistance determinants. Despite the near total elimination of IPD caused by serotypes covered by the vaccine, significant disease burden still exists globally owing to the emergence of serotypes not included in PCV7, particularly 19A. Serotype shifts have led to the development of a newer vaccine, PCV13, which covers six additional serotypes, including 19A. In addition to the capsule, pili (type 1 pili and type 2 pili) have recently been described as virulence factors of S. pneumoniae and have been shown to mediate critical host–bacteria interactions such as adhesion to human epithelium, facilitate interaction with extracellular matrix proteins and provide a pro-inflammatory stimulus.

The objective of the current study was to characterize respiratory isolates of MRSP obtained from patient samples collected across Canada between 1998 and 2008. Our characterization involved detection of MDR, study of the mechanisms of macrolide resistance, study of genetic relatedness, serotyping and determining PCV7 coverage, as well as the detection of newly described pili virulence factors.

Materials and methods

S. pneumoniae isolates

S. pneumoniae isolates (n = 12,759) were collected between September 1998 and December 2006 as part of the CROSS study (Canadian Respiratory Organism Susceptibility Study) and between January 2007 and December 2008 as part of the CANWARD study (Canadian Ward Surveillance Study). Participating medical centres in both studies were analogous, represented all regions of Canada and sent consecutive lower respiratory tract isolates of S. pneumoniae to the Health Sciences Centre in Winnipeg, Canada (one isolate per patient). The identity of each isolate was confirmed by methods recommended by the CLSI. Isolates confirmed to be S. pneumoniae were stocked in skimmed milk and stored at −80°C prior to further testing.

Antimicrobial susceptibility testing

MICs were determined using the broth microdilution method according to CLSI guidelines using custom-designed, in-house prepared, 96-well microtitre panels containing doubling dilutions of antimicrobial agents in 100 µL of cation-adjusted Mueller–Hinton broth supplemented with 4% lysed horse blood. CLSI breakpoints were used to interpret MICs. Isolates with an erythromycin (clarithromycin) MIC of ≥1 mg/L were defined as macrolide resistant. Penicillin-resistant S. pneumoniae was defined using the oral penicillin V CLSI breakpoint of ≥2 mg/L. Inducible clindamycin resistance (IMLSB) was determined using the CLSI disc approximation test (D test) for all erythromycin-resistant, clindamycin-susceptible isolates of S. pneumoniae with an erm(B) genotype.

Determination of macrolide resistance mechanisms

Macrolide-resistant isolates were analysed for the presence of the erm(B) and mef(A) genes using a previously described PCR assay.

PFGE

The genetic relatedness of isolates of S. pneumoniae was studied using a previously described PFGE method. PFGE testing was restricted to MRSP isolates that were dual mef(A) and erm(B) positive.

Serotyping and PCV7 coverage

S. pneumoniae isolates were serotyped using the Quellung reaction with antisera obtained from the Statens Serum Institut (Copenhagen, Denmark). A PCR-based molecular serotyping method was used to differentiate between serotypes 6A and 6C. Vaccine coverage was assessed by grouping pneumococcal serotypes into PCV7 serotypes (4, 6B, 9V, 14, 18C, 19F and 23F), PCV7-related serotypes (6A, 9A, 9L, 9N, 18F, 19B, 19C, 23A and 23B) and non-PCV7 serotypes (19A and all other serotypes).

Pili detection

The detection of type 1 and type 2 pili was carried out by PCR using a previously described method. The isolates tested for the presence of pili virulence factors were restricted to those MRSP with a 19F or 19A serotype and those that were dual mef(A) and erm(B) positive, as both of these isolate types emerged over the course of the study.

Statistical analysis

Differences in the proportions among categorical variables were assessed using Fisher’s exact test or the χ² test, as appropriate. A P value of ≤0.05 was considered to be statistically significant. All analyses were performed using JMP® software version 10 (SAS Institute Inc., Cary, NC, USA).

Results

Demographic characteristics associated with MRSP

In total, 1518 respiratory MRSP isolates were collected as part of the CROSS and CANWARD surveillance studies. The prevalence of MRSP remained at 8% from 1998 to 2001, increased to 15% by 2003 and to 22% by 2008 (P = 0.0001, 1998 versus 2008). MRSP were isolated primarily from patients aged 18–64 years (46% of isolates) and ≥65 years (35% of isolates). When isolates from 1998 (n = 70) and 2008 (n = 84) were compared, significant changes in the percentage of MRSP isolated were noted for patients aged <2 years (8.6% and 0%, respectively; P = 0.0002), 5–17 years (4.3% and 6.0%, respectively; P = 0.04) and 18–64 years (48.6% and 53.6%, respectively; P = 0.03) and for the percentage of MRSP isolated from patients in British Columbia/Alberta (42.9% and 9.5%, respectively; P = 0.003), Saskatchewan/Manitoba (15.7% and 21.4%, respectively; P = 0.03) and Ontario (5.7% and 29.8%, respectively; P = 0.002). Throughout the study, 60% of isolates came from male patients and 40% from outpatients.
Characteristics of macrolide resistance mechanisms

Of the 1518 isolates of MRSP, 51% carried the \( \textit{mef}(A) \) gene alone, 36% carried the \( \textit{erm}(B) \) gene alone, 8% carried both the \( \textit{mef}(A) \) and \( \textit{erm}(B) \) genes and 5% did not carry either gene (Figure 1). Isolates carrying the \( \textit{mef}(A) \) gene alone ranged from 43% in 2001 to 59% in 2006 and decreased during the study period from 55% in 1998 to 50% in 2008 (\( P = 0.037 \)). Isolates carrying the \( \textit{erm}(B) \) gene alone ranged from 27% in 2005 to 51% in 2001 and decreased during the study period from 41% in 1999 to 19% in 2008 (\( P = 0.015 \)). The presence of both the \( \textit{mef}(A) \) and \( \textit{erm}(B) \) genes among MRSP ranged from 0.8% in 1999 to 19% in 2008 and increased during the study period from 3% in 1998 to 19% in 2008 (\( P = 0.001 \)). MRSP isolates not carrying either the \( \textit{mef}(A) \) or \( \textit{erm}(B) \) gene varied from 1% in 1999 to 11% in 2005 and increased during the study period from 1.8% in 1998 to 3.6% in 2008 (\( P = 0.0365 \)).

The antimicrobial susceptibilities of MRSP stratified by genotype are shown in Table 1. \( \textit{mef}(A) \)-positive isolates demonstrated lower macrolide MICs (MIC\( _{90} \) 4 mg/L) than \( \textit{erm}(B) \)-positive isolates (MIC\( _{90} \) >64 mg/L); however, some \( \textit{mef}(A) \)-positive isolates exhibited higher MICs than expected (16–64 mg/L). The majority (98%) of \( \textit{mef}(A) \)-positive isolates were susceptible to clindamycin (MIC\( _{90} \) ≤0.25 mg/L), whereas the majority (90%) of \( \textit{erm}(B) \)-positive isolates were resistant to clindamycin (MIC\( _{90} \) >16 mg/L). Of the 59 clindamycin-susceptible \( \textit{erm}(B) \)-positive isolates, 27 (46%) were shown to exhibit inducible clindamycin (iMLSB) resistance using the D test.

All isolates were analysed for MDR phenotypes, with 24% of MRSP isolates being co-resistant to penicillin, and 16% co-resistant to both penicillin and trimethoprim/sulfamethoxazole. Among \( \textit{erm}(B) \)-positive, \( \textit{mef}(A) \)-positive, \( \textit{erm}(A) \)- and \( \textit{erm}(B) \)-negative, and dual \( \textit{mef}(A) \)- and \( \textit{erm}(B) \)-positive isolates of MRSP, 20% and 10%, 24% and 14%, 11% and 6%, and 66% and 53% of isolates were co-resistant to penicillin, and to both penicillin and trimethoprim/sulfamethoxazole, respectively.

Serotype distribution and PCV7 vaccine coverage among MRSP

Forty-one different serotypes were identified among the 1518 isolates of MRSP. The serotypes (% of isolates) were: 3 (0.9%), 4 (0.1%), 6A (6.3%), 6B (14.8%), 6C (0.7%), 7F (0.1%), 8 (0.1%), 9A (0.7%), 9L (0.1%), 9N (0.6%), 9V (4.5%), 11A (2.8%), 12B (0.1%), 12F (3.6%), 14 (7.7%), 15A (3.8%), 15B (2.8%), 15C (2%), 15F (0.1%), 16 (0.1%), 17F (0.1%), 18B (0.1%), 18C (0.7%), 19A (4.1%), 19B (0.1%), 19F (16%), 20 (0.1%), 21 (0.1%), 22F (0.9%), 23A (0.4%), 23B (0.2%), 23F (11.4%), 29 (0.2%), 31 (1%), 33 (0.1%), 33B (0.5%), 33F (1.9%), 34 (0.5%), 35A (0.5%), 35B (0.9%), 35F (0.5%) and non-typeable (9.5%). Overall, serotype 19F was the predominant serotype, followed by 6B, 23F and non-typeable.

The prevalence of PCV7 serotypes during the course of the study is shown in Figure 2. Overall, 56% of isolates had a PCV7 serotype. Five (19F, 6B, 23F, 14 and 9V) of the seven PCV7 serotypes were among the 10 most common serotypes. The prevalence of serotypes 6B, 9V, 14, 18C, 19F and 23F decreased by 5%, 7%, 7%, 0%, 16% and 6%, respectively, between 1998 and 2008. The prevalence of isolates with six (6B, 9V, 14, 18C, 19F and 23F) of the seven PCV7 serotypes decreased (by 2%, 3%, 7%, 1%, 5% and 11%) between the periods before (1998–2000) and after (2005–08) PCV7 introduction in Canada (Figure 2a). The percentage of isolates covered by PCV7 ranged from a high of 74% (2000 and 2001) to a low of 18% (2007). Isolates covered by PCV7 decreased by 36%, from 67% in 1998 to 31% in 2008 (Figure 2b).

Overall, 36% of isolates had a non-PCV7 serotype. The most prevalent (emerging) non-PCV7 serotypes were: 6A (6.3%), 19A
Isolates with these serotypes increased in prevalence by 4%, 5%, 3%, 0%, 5%, 3%, 4% and 3%, respectively, between the periods before (1998–2000) and after (2005–08) PCV7 introduction in Canada. A 15% increase was observed among isolates with serotype 19A between 1998 (1%) and 2008 (15.5%) of the study (P = 0.0145). This increase was observed among all age groups, but predominantly (84%) among elderly and adult populations. Isolates not covered by PCV7 increased from 33% in 1998 to 57% in 2008 (Figure 2b).

**Table 1. Antimicrobial susceptibilities of 1518 MRSP with different genotypes**

<table>
<thead>
<tr>
<th>Organism/genotype (n), antimicrobial agent</th>
<th>MIC (mg/L)</th>
<th>MIC interpretation</th>
</tr>
</thead>
<tbody>
<tr>
<td>All MRSP (1518)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>erythromycin</td>
<td>8</td>
<td>1 to &gt;64</td>
</tr>
<tr>
<td>clindamycin</td>
<td>≤0.25</td>
<td>≤0.25 to &gt;16</td>
</tr>
<tr>
<td>penicillina</td>
<td>0.25</td>
<td>0.03–8</td>
</tr>
<tr>
<td>trimethoprim/sulfamethoxazole</td>
<td>1</td>
<td>0.12–16</td>
</tr>
<tr>
<td>doxycycline</td>
<td>1</td>
<td>0.25–32</td>
</tr>
<tr>
<td>erm(B)-positive MRSP (546)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>erythromycin</td>
<td>&gt;64</td>
<td>1 to &gt;64</td>
</tr>
<tr>
<td>clindamycin</td>
<td>&gt;16</td>
<td>≤0.25 to &gt;16</td>
</tr>
<tr>
<td>penicillin</td>
<td>0.25</td>
<td>0.03–8</td>
</tr>
<tr>
<td>trimethoprim/sulfamethoxazole</td>
<td>1</td>
<td>0.12–16</td>
</tr>
<tr>
<td>doxycycline</td>
<td>4</td>
<td>0.25–32</td>
</tr>
<tr>
<td>mef(A)-positive MRSP (776)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>erythromycin</td>
<td>4</td>
<td>1–64</td>
</tr>
<tr>
<td>clindamycin</td>
<td>≤0.25</td>
<td>≤0.25 to &gt;16</td>
</tr>
<tr>
<td>penicillin</td>
<td>0.12</td>
<td>0.03–8</td>
</tr>
<tr>
<td>trimethoprim/sulfamethoxazole</td>
<td>0.5</td>
<td>0.12–16</td>
</tr>
<tr>
<td>doxycycline</td>
<td>0.25</td>
<td>0.25–32</td>
</tr>
<tr>
<td>mef(A)-positive + erm(B)-positive MRSP (116)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>erythromycin</td>
<td>64</td>
<td>1–64</td>
</tr>
<tr>
<td>clindamycin</td>
<td>16</td>
<td>0.12–16</td>
</tr>
<tr>
<td>penicillin</td>
<td>4</td>
<td>0.03–8</td>
</tr>
<tr>
<td>trimethoprim/sulfamethoxazole</td>
<td>8</td>
<td>0.12–16</td>
</tr>
<tr>
<td>doxycycline</td>
<td>2</td>
<td>0.25–32</td>
</tr>
<tr>
<td>mef(A)-negative + erm(B)-negative MRSP (80)</td>
<td></td>
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<tr>
<td>erythromycin</td>
<td>16</td>
<td>1–64</td>
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<tr>
<td>clindamycin</td>
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<td>0.12–16</td>
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<td>penicillin</td>
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<tr>
<td>trimethoprim/sulfamethoxazole</td>
<td>0.25</td>
<td>0.12–16</td>
</tr>
<tr>
<td>doxycycline</td>
<td>0.25</td>
<td>0.25–32</td>
</tr>
</tbody>
</table>

S, susceptible; I, intermediate; R, resistant.

aPenicillin MIC interpretation based on oral penicillin V CLSI breakpoints: S, ≤0.06 mg/L; I, 0.12–1 mg/L; R, ≥2 mg/L.

(4.1%), 15A (3.8%), 12F (3.6%), 15B (2.8%), 11A (2.8%), 15C (2%) and 33F (1.9%) (Figure 2c). Isolates with these serotypes increased in prevalence by 4%, 5%, 3%, 0%, 5%, 3% and 0%, respectively, between the periods before (1998–2000) and after (2005–08) PCV7 introduction in Canada. A 15% increase was observed among isolates with serotype 19A between 1998 (1%) and 2008 (15.5%) of the study (P = 0.0145). This increase was observed among all age groups, but predominantly (84%) among elderly and adult populations. Isolates not covered by PCV7 increased from 33% in 1998 to 57% in 2008 (Figure 2b).

PCV7 coverage was analysed by genotype. Overall, PCV7 coverage was 47%, 50%, 64% and 66% for MRSP isolates with a genotype that was erm(B) and mef(A) negative, mef(A) positive alone, erm(B) positive alone and dual erm(B) and mef(A) positive, respectively. PCV7 coverage decreased by 80%, from 100% in 1999 to 20% in 2008, among isolates with a dual erm(B)- and mef(A)-positive genotype. The percentage of dual erm(B)- and mef(A)-positive isolates not covered by PCV7 averaged 25% per year from 1998 to 2006 and 75% per year in 2007 and 2008. Among serogroup 19 isolates, the ratio of serotype 19A to serotype 19F (19A:19F) changed from 1:8 in 1998–2006 to 1:3 in 2007–08.

Detection of pili virulence factors in isolates of MRSP with a 19A or 19F serotype and/or a dual mef(A)- and erm(B)-positive genotype

As described earlier, the prevalence of dual mef(A)- and erm(B)-positive MRSP increased by 16% from 1998 (3%) to 2008 (19%). 60% of these isolates were serotype 19F and 12% were serotype 19A. In total, 82 of the 116 MRSP that were positive for both mef(A) and erm(B) were serotype 19F or 19A. Serotype 19A, a non-PCV7 serotype, emerged among dual erm(B)- and mef(A)-positive isolates in 2003 (8%) and increased to >50% of dual-positive
MRSP by 2008 (range, 5% in 2004 to 63% in 2007). Isolates of serotype 19A were shown to have a high degree of genetic relatedness by PFGE (data not shown) and to be MDR (penicillin non-susceptibility rate, 100%; trimethoprim/sulfamethoxazole non-susceptibility rate, 100%; clindamycin non-susceptibility rate, 100%; doxycycline non-susceptibility rate, 86%). The presence of pili virulence factors among MRSP isolates of serotype 19F or 19A and/or a dual mef(A)- and erm(B)-positive genotype are summarized in Table 2. The presence of genes for type 1 pili and type 2 pili were significantly greater (P < 0.0001) among MRSP with a 19F or 19A serotype and dual positivity for both the mef(A) and erm(B) genes.

Discussion

In our 11 year study, the prevalence of MRSP increased from 8% in 1998 to 22% in 2008. Increases in macrolide resistance have been linked to increased overall consumption of macrolides and the use of longer-acting macrolides such as clarithromycin and azithromycin.22,23

Efflux-mediated resistance remained the most common mechanism during our study; however, it decreased slightly from 55% in 1998 to 50% in 2008. The decline in efflux-mediated MRSP in our study is not as pronounced as the decrease of >10% reported by US investigators.24 Isolates with efflux-mediated macrolide resistance may still be successfully treated with macrolides as their usual MICs (1–16 mg/L) are in the range of concentrations (at the site of infection) achievable by most macrolides following standard dosages.25 Therefore, the decline in MRSP isolates with efflux-mediated resistance may be of clinical relevance, especially if the isolates are being replaced with others possessing an erm(B) gene (MIC ≥32 mg/L) or mutationally based resistance. Furthermore, some mef(A)-carrying S. pneumoniae have been shown to have higher than usual macrolide MICs (16–64 mg/L).26 This is important because pharmacodynamic and animal models of pneumococcal pneumonia have shown that macrolides are unable to eradicate mef(A)-carrying isolates with MICs >16 mg/L.25 In contrast to studies published by investigators in the USA, mef(A)-positive isolates with macrolide MICs of 16–64 mg/L in the current study were sporadic and did not show an increase over time.

Isolates carrying both the mef(A) and erm(B) genes increased by 16% from 3% in 1998 to 19% in 2008 throughout our study. The emergence of dual mef(A)- and erm(B)-positive isolates has been observed worldwide.6,7,18 The majority of dual-positive isolates in the current study were MDR (resistant to MLSB antimicrobials, penicillin, trimethoprim/sulfamethoxazole and doxycycline), genetically related (by PFGE) and serotypically related (19F or 19A).

The emergence and increasing prevalence of these isolates may be attributed to their MDR phenotype and the implementation of PCV7.26,27 The incidence of dual erm(B)- and mef(A)-positive MRSP has been reported to have increased in the years following PCV7 implementation as a result of a 19F to 19A serotype switch.26,27 In the current study, we observed serotype 19A emerge among the dual erm(B)- and mef(A)-positive MRSP isolates in 2008; by 2007 it was more common than 19F and in 2008 it became the predominant dual erm(B)- and mef(A)-positive serotype.

The current study summarized the serotype distribution and PCV7 coverage among MRSP in Canada between 1998 and 2008. During the study, the prevalence of PCV7 serotypes decreased by 36%, from 67% in 1998 to 31% in 2008. The greatest decrease was noted among isolates with serotype 19F (16%; however, it was still present in significant numbers in the last year of the study, probably due to the low immunogenicity of serotype 19F.28 Among the emerging serotypes (6A, 19A, 15A, 12F, 15B, 11A, 15C and 33F) the most concerning was 19A. It increased across all age groups, with the highest increase (60%) noted for the adult (18–64 years), primarily unvaccinated, age group (P < 0.05). The emergence of 19A among the adult population is especially concerning as this age group is generally considered to be at lower risk for pneumococcal infections than other groups (unless comorbidity or immunosuppression exists). More importantly, isolates with serotype 19A increased from 8% (2003) to over 50% (2008) among MDR dual mef(A)- and erm(B)-positive isolates and were largely clonal by PFGE analysis and genetically related to isolates with serotype 19F.

Although serotype 6A was the most common among the emerging serotypes, 9.5% of serotype 6A isolates identified by the Quellung reaction were serotype 6C by PCR.29,30 Among the non-vaccine serotypes, serotypes 1, 3, 7F and 22F have been described in the literature as emerging since the implementation of PCV7.31,32 During this study, serotypes 3 and 22F were detected in 10 isolates each (0.0% of MRSP). One isolate of serotype 7F was found and no serotype 1 isolates were detected. Low numbers of these serotypes in this study might reflect the invasive nature of these serotypes and that the current study focused on respiratory isolates.12

Because the PCV13 vaccine, providing coverage against six additional serotypes (1, 3, 4, 5, 6A, 7F and 19A), was implemented into the routine childhood programme in Canada in 2010, the serotype distribution in this study does not reflect its use.13 However, retrospective analysis allows the use of the data as a baseline from which changes can be assessed. The retrospective analysis showed that PCV13 would have provided coverage for 68% of

<table>
<thead>
<tr>
<th>MRSP type</th>
<th>Pili gene prevalence, %</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>type 1</td>
</tr>
<tr>
<td>dual erm(B)- and mef(A)-positive genotype</td>
<td>present</td>
</tr>
<tr>
<td>19F or 19A serotype</td>
<td>present</td>
</tr>
<tr>
<td>Isolates, n</td>
<td>82</td>
</tr>
<tr>
<td>19F or 19A serotype</td>
<td>absent</td>
</tr>
</tbody>
</table>
isolates. The coverage ranged from a high of 80% in 1999 to a low of 50% in 2007. The PCV13 vaccine coverage decreased by 30% over the course of the study. Future studies will determine the effectiveness of the PCV13 vaccine and are imperative in determining the evolution of serotypes among S. pneumoniae.

Pili have recently been recognized in S. pneumoniae and are implicated in its virulence. Pili have been shown to be associated with isolates belonging to capsular serotypes included in PCV7 such as 19F. Consequently, the initial decline in vaccine serotypes following the introduction of PCV7 produced a decline in the prevalence of pilated S. pneumoniae. However, the re-emergence of pili among S. pneumoniae with non-vaccine serotypes has been reported. In the current study, we investigated the presence of pili among the emerging MDR, dual erm(B)- and mef(A)-positive, genetically related 19A and 19F isolates of S. pneumoniae. We showed that these isolates are strongly associated with the presence of pili. This observation seems to contradict the dictum that bacteria become less virulent as they become more antimicrobial resistant; this frequently observed relationship can be seen by assessing the antimicrobial susceptibility profiles for invasive versus non-invasive isolates of pneumococci. Whether pili are responsible for more complicated disease processes or whether they provide an evolutionary advantage to these isolates that allows them to spread despite vaccine pressure remains unknown.

In conclusion, our study demonstrates that MRSP isolated in Canadian hospital laboratories are increasingly MDR, genetically related, pilated and frequently consist of serotypes not found in PCV7. From 1998 to 2008, respiratory isolates of MRSP in Canada increased significantly due primarily to the emergence and spread of serotypes 6A, 19A and other non-PCV7 serotypes.

Acknowledgements
We would like to thank Barbara Weshnowski, Ravinder Vashisht, Nancy Laing and Frani Tailor for technical assistance. More information regarding the Canadian Antimicrobial Resistance Alliance (CARA) and its members can be found at www.can-r.ca, the official web site of CARA.

Funding
This work was supported in part by the University of Manitoba, Health Sciences Centre (Winnipeg, Manitoba, Canada) and the National Microbiology Laboratory (Winnipeg, Manitoba, Canada) as well as by Abbott Laboratories Ltd, Affinium Pharmaceuticals Inc., Astellas Pharma Canada Inc., AstraZeneca, Bayer Canada, Cerexa Inc./Forest Laboratories Inc., Cubist Pharmaceuticals, Merck Frosst, Pfizer Canada Inc., Sunovion Pharmaceuticals Canada Inc. and The Medicines Company.

Transparency declarations
D. J. H. and G. G. Z. have received research funding from the above-mentioned companies. All other authors have no declarations.

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