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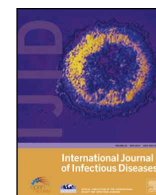

Pertactin-negative *Bordetella pertussis* strains in Canada: characterization of a dozen isolates based on a survey of 224 samples collected in different parts of the country over the last 20 years

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SUMMARY

Objective: To detect and characterize pertactin-negative *Bordetella pertussis* in Canada, especially for isolates collected in recent years.
Methods: A total of 224 isolates from the years 1994–2013 were screened by Western immuno-blot for expression of pertactin. Pertactin-negative isolates were characterized by serotyping, pulsed-field gel electrophoresis (PFGE), and genotyping of their pertactin, fimbriae 3, pertussis toxin subunit 1, and pertussis toxin gene promoter region, as well as the complete sequence of the pertactin gene.
Results: Twelve isolates were pertactin-negative, giving an overall prevalence of 5.4%. However, no such isolate was found prior to 2011 and 17.8% of 62 isolates examined in 2012 were pertactin-negative. Ten pertactin-negative isolates contained a significant mutation in their pertactin (*prn*) genes. IS*481* was found in the *prn* genes of eight isolates, while a single point mutation occurred either in the coding region (resulting in a premature stop codon) or in the promoter region (preventing gene transcription) in two other isolates. PFGE analysis also showed multiple profiles suggesting that several independent genetic events might have led to the emergence of these pertactin-negative strains rather than expansion of a single clone.
Conclusions: As reported elsewhere, pertactin-negative *B. pertussis* has emerged in Canada in recent years, notably in 2012. This coincided with an increase in pertussis activity in Canada. A further systematic study with a larger geographical representative sample is required to determine how these vaccine-negative strains may contribute to the overall changing epidemiology of pertussis in Canada.
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1. Introduction

Whooping cough, or 100 days cough in Chinese, is caused by the bacterium *Bordetella pertussis*. Although whooping cough used to be mainly a childhood disease, it now also occurs in adolescents and adults, with a worldwide distribution. A pertussis vaccine was introduced in Canada as early as 1943. Unlike some vaccines that target only certain serotypes or serogroups, both the whole-cell and the acellular pertussis vaccines are believed to be universally effective in preventing pertussis caused by all strains of *B. pertussis*. Despite this fact and the long history of immunization programs in many countries, pertussis continues to be a vaccine-preventable disease with a high number of cases and outbreaks reported periodically – as recently as 2012 in the USA, UK, Australia, Canada[1–4] – resulting in deaths in infants too young to be immunized or those who have not completed the schedule of all

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doses of vaccine. In the UK, maternal immunization has been introduced as an indirect way to protect the newborn and infants.[5]

Research in areas to improve immune-protection, such as changing the current vaccine formula to increase its immunogenicity and duration of protection, altering the schedules of immunization in order to optimize the protection period, and even the development of newer vaccines, are all being examined. These suggested changes/interventions are based on the premises that recent observations have suggested the inferior immunogenicity of the acellular pertussis vaccine versus the pertussis whole-cell vaccine,[6] and the rapid decline of immunity offered by booster doses of acellular vaccines.[7,8]

Potential reasons for the continued presence of pertussis disease activity in highly vaccinated populations have been discussed elsewhere and include increased awareness and testing by front-line health care providers, improved laboratory diagnostics, waning vaccine immunity, suboptimal acellular pertussis vaccines, and changes in the *B. pertussis* bacterial population. Strains that produce an increased amount of pertussis toxin such as the ptxP3 strain[9,10] and strains that do not have a perfect match with the vaccine (lacking or not expressing vaccine components) have been described.[11–16]

After decades of pertussis vaccination programs in infants, followed by the more recently introduced acellular pertussis vaccines in school-aged children, adolescents, and adults, the incidence of pertussis in Canada reached a record low of 2 cases per 100 000 population in 2011.[2] However, a sharp increase in pertussis incidence (13.9 per 100 000 population) was reported in Canada in 2012 due to increases in reported cases in nine different provinces and territories (British Columbia, Alberta, Manitoba, Ontario, Québec, New Brunswick, Nova Scotia, Prince Edward Island, and Yukon),[2] with some populations reporting the highest incidence rates in North America.[17]

Although Canada does not have a formal laboratory surveillance program for pertussis, the National Microbiology Laboratory (NML) carries out periodic studies in collaboration with provinces and territories to examine strain characteristics.[13,18,19] The molecular characteristics of Canadian pertussis strains are similar to those described in other countries.[3,9,14–16] First detected and reported in France in 2007,[12] vaccine antigen-negative *B. pertussis* strains, particularly pertactin-deficient strains, have now been observed in different parts of the world,[20–23] including the USA.[24] The objectives of this article are to report on pertactin-negative *B. pertussis* isolates in Canada and the characteristics of these isolates, as well as how they may contribute towards the overall epidemiology of pertussis in Canada.

2. Materials and methods

2.1. Bacterial standard strains and clinical isolates

Reference strains for studies on pertactin expression were obtained from the American Culture Collection (Rockville, MD, USA). *B pertussis* clinical isolates were provided by provincial public health laboratories for serotyping and genetic characterization either as part of a planned study,[13,18,19] or as ad hoc activity for outbreak investigation (see also the 'Results and discussion' section). Only isolates from the culture collection of the NML were included in this study. These included 100% of the *B. pertussis* collection from recent years (2007–2013), although only small numbers were tested in each of those years (ranging from 5 in 2013 to 62 in 2012). Nevertheless, outbreak strains from British Columbia (2012) and Saskatchewan (2010) were included. The number of isolates tested per year during the period 1994–2006 ranged from a low of 2 in 2000 to 19 in 2005. A list of the strains included in this study can be found in the **Supplementary Material** (Table S1).

In total we examined 224 *B. pertussis* isolates collected in different parts of Canada between 1994 and 2013. Sixty-nine isolates were from the period 1994–2005 and represented 12.3% of the NML *B. pertussis* collection from that period. One hundred and forty-two isolates were from the years 2006–2013 and represented 98.6% of the NML *B. pertussis* collection from that period. The year of isolation was not available for 13 of the tested isolates.

2.2. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western immuno-blot

Western immuno-blot analysis was adapted from previously described procedures,[23,25] using sheep anti-pertactin antiserum purchased from The National Institute of Biological Standards and Control (Hertfordshire, UK). *B. pertussis* cells from approximately one-eighth of a loopful of a 48-h culture on a Bordet Gengou agar plate were suspended in 200 μl phosphate buffered saline (PBS). Ten microlitres of the cell suspension was added to 10 μl of SDS-PAGE sample buffer (Bio-Rad Canada, Montreal, Quebec, Canada), and this was heated at 95 °C for 10 min. The lysate was separated using SDS-PAGE. Samples were run against a pre-stained Precision Plus Protein Dual Color Ladder (BioRad Canada), with a positive control strain (*B. pertussis* ATCC 9797) and a negative control (*Bordetella avium* ATCC 35086). Proteins resolved by SDS-PAGE were transferred onto a polyvinylidene difluoride (PVDF) membrane, which was blocked overnight with 5% skim milk in PBS, then incubated with anti-pertactin antibody, diluted 1:1000, for 2 h at 37 °C. Membranes were washed with PBS with 0.05% Tween 20 (Sigma Chemicals, St. Louis, USA) (PBST), then incubated with a secondary donkey anti-sheep horseradish peroxidase (HRP)-conjugated antibody (Jackson ImmunoResearch Laboratories Inc., West Grove, PA, USA), diluted 1:1000, for 1 h at room temperature. After a final wash of four times with PBST, membranes were developed with Metal Enhanced DAB Substrate (Thermo Fisher Scientific, Waltham, MA, USA).

2.3. DNA sequencing

The pertactin genes in isolates determined to be pertactin-negative by Western immuno-blot were sequenced using a combination of in-house and previously described primer sets.[24,26] The complete set of PCR primers and PCR conditions are available from the authors. Sequences were analyzed using DNASTAR software (DNASTAR Inc., Madison, WI, USA) and compared to the *B. pertussis prn2* gene sequence of strain B345 (GenBank accession number **AJ011092**).[27]

2.4. Serotyping and genotyping

Serotyping was carried out by indirect whole-cell ELISA, as described previously.[28] Partial gene sequences of the pertussis toxin subunit 1 (*ptxS1*), pertactin region 1 (*prn*), fimbriae 3 (*fim3*), and pertussis toxin promoter region (*ptxP*) were performed as described before.[19] Sequence types (STs) were defined based on the combination of alleles of the *ptxS1*, *prn*, *fim3*, and *ptxP*, as described previously.[19]

3. Results and discussion

Of the 224 isolates examined by Western immuno-blot for expression of pertactin, only 12 (5.4%) were found not to express pertactin. A representative immuno-blot depicting positive and negative results is shown in Figure 1. Of these 12 pertactin-negative isolates, 11 were isolated in 2012 and one in 2011. Five of

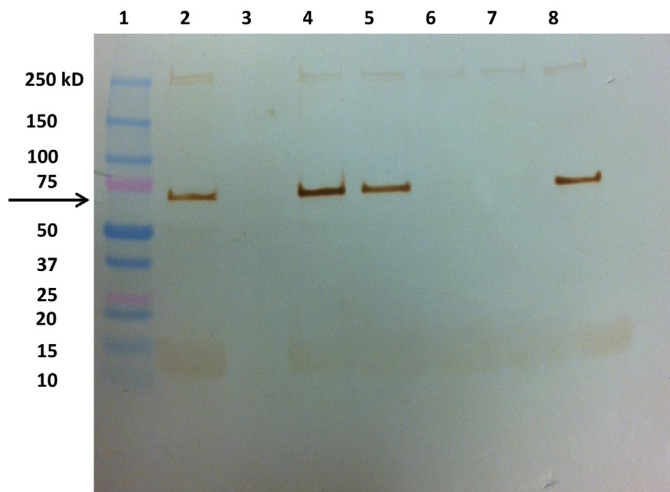

Figure 1. Representative Western immuno-blot to detect the expression of pertactin antigen in *Bordetella pertussis* isolates in Canada. Lane 1, Precision Plus Protein Dual Color Ladder with the molecular weight of bands marked to the left; lane 2, *B. pertussis* ATCC 9797, positive control; lane 3, *Bordetella avium* ATCC 35086, negative control; lanes 4–8, test samples (lanes 4, 5, and 8 show positive results for pertactin expression, while lanes 6 and 7 show negative results for pertactin). Arrow indicates the position of pertactin in the Western immuno-blot.

the pertactin-negative isolates were from the province of Alberta (all in 2012), while seven were from the province of Ontario. Five of the pertactin-negative *B. pertussis* isolates belong to ST-1, characterized by the genotype *ptxS1A*, *prn2*, *fim3B*, and *ptxP3*, while seven belonged to ST-2, characterized by the genotype *ptxS1A*, *prn2*, *fim3A*, and *ptxP3*. Isolates belonging to ST-1 and ST-2 were seen in both provinces. All 12 pertactin-negative isolates expressed the Fim3 but not the Fim2 serotype antigen.

A genetic mutation that explained the lack of pertactin expression was found in 10 of the pertactin-negative isolates (Table 1). IS*481* insertion sequences were found in eight strains (66.7%), with seven located within the coding region at nucleotide position 1613, relative to the *prn2* gene sequence of *B. pertussis* strain B345 (GenBank accession number **AJ011092.1**), and in one strain at nucleotide position 245. In six isolates the IS elements were found within the *prn2* gene in the reverse direction, while in two isolates they were found in the forward direction (Figure 2), as observed previously by others (GenBank accession numbers **AB670736** and **AB670737**).

Besides the common mechanism of disruption of the *prn* gene due to IS*481*, we found one isolate with a cytosine to thymine mutation at position 1273, resulting in a premature stop codon at amino acid position 425, which has also been observed by others.[24] In another isolate, a guanine to adenine mutation was found in the promoter region at position −162, suspected to disrupt transcription of the gene. The mutation site identified in this strain was equivalent to the putative −10 promoter sequence identified by Kinnear et al.,[29] to which the RNA polymerase was expected to bind in the promoter. In the remaining two pertactin-negative isolates, no mutation was found in their *prn* genes, neither in the promoter region nor in the coding sequence, suggesting other potential genetic defects not identified in this study.

To further define the genetic relatedness of the pertactin-negative *B. pertussis* isolates, PFGE profiles obtained using two restriction enzymes were analysed. With SpeI restriction enzyme, eight different profiles were obtained, while with XbaI, five profiles were observed. When PFGE patterns generated by the two enzymes were combined, 10 restriction profiles were obtained (Figure 3).

The epidemiology of pertussis in Canada is complex, with widely varying rates found in the different provinces and territories.[2] This may be due in part to the different vaccine products used as well as the different introduction dates and schedules implemented by the different provinces and territories.[30,31] Currently only two kinds of acellular pertussis vaccine are licensed for use in Canada (http://www.phac-aspc.gc.ca/publicat/cig-gci/introduction-eng.php). One form consists of five components (pertussis toxoid, pertactin, filamentous hemagglutinin, fimbriae 2, and fimbriae 3), while the other form consists of three components (pertussis toxoid, pertactin, and filamentous hemagglutinin). Also, each acellular vaccine should be considered a different product as different vaccines may have varying concentrations of pertactin as well as other targets (http://www.who.int/biologicals/areas/vaccines/apertussis/en/).

The overall prevalence of the pertactin-deficient phenotype among *B. pertussis* isolates examined in Canada was 5.4% (12/224 isolates). In 2011, the prevalence was 10% (1/10 isolates) and this increased to 17.8% (11/62 isolates) in 2012. Based on this study, pertactin-negative strains were only found in the provinces of Alberta and Ontario since 2011 and mainly in 2012. The percentage of this phenotype among the isolates tested in these two provinces in 2012 increased to 9.6% and 60% for Alberta and Ontario, respectively. This sudden increase in the finding of pertactin-deficient *B. pertussis* isolates appears to coincide with increases in pertussis activity in a number of provinces in Canada. However, the pertactin-deficient phenotype is notably absent from the outbreak strains in Saskatchewan (2010) and British Columbia (2012), albeit the total number of isolates examined from these outbreaks were small (five in Saskatchewan and nine in British Columbia). Whether the pertactin-deficient phenotype might have contributed to the outbreaks in Ontario and Alberta would require further in-depth studies matching clinical and epidemiological data with results from the laboratory characterization of strains.

Another feature of the pertactin-negative *B. pertussis* isolates is the fact that they all have the genotype *ptxS1A*, *ptxP3*, and *prn2*. However, their *fim3* gene sequences divide them into two

Table 1
Characteristics of 12 pertactin-deficient *Bordetella pertussis* isolates from the provinces of Alberta (AB) and Ontario (ON) in 2011 and 2012

Number of isolates	Year of isolation	Province	*prn* mutation and nucleotide position[a]	Sequence type	PFGE profile
1	2012	ON	No mutations	ST-1	Xba 076 Spe 001
1	2012	AB	No mutations	ST-2	Xba 021 Spe 003
1	2011	ON	G to A mutation in promoter region, position −162	ST-2	Xba 071 Spe 056
1	2012	ON	C to T mutation at position 1273, resulting in stop codon	ST-2	Xba 021 Spe 001
1	2012	AB	IS*481* inverted at position 245	ST-2	Xba 077 Spe 064
1	2012	AB	IS*481* at position 1613	ST-2	Xba 073 Spe 065
1	2012	ON	IS*481* at position 1613	ST-2	Xba 073 Spe 001
1	2012	AB	IS*481* inverted at position 1613	ST-2	Xba 021 Spe 020
1	2012	AB	IS*481* inverted at position 1613	ST-1	Xba 076 Spe 038
3	2012	ON	IS*481* inverted at position 1613	ST-1	Xba 076 Spe 004

PFGE, pulsed-field gel electrophoresis.
[a] Position relative to the *prn2* gene of *B. pertussis* strain B345 (**AJ011092**).

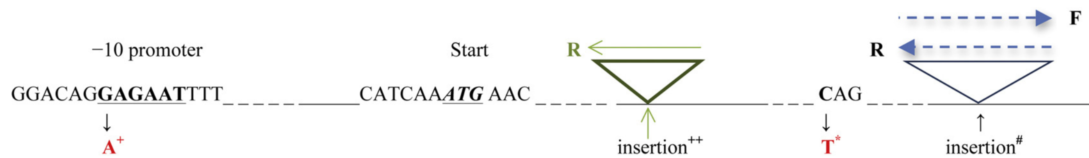

Figure 2. Schematic diagram of the *prn* gene showing the various types of mutation that affect expression of the pertactin antigen. Locations of the start codon (marked in bold italic and underlined) and the −10 promoter sequence (marked in bold and underlined) are shown (adapted from Kinnear et al., 1999[29]). +Guanine to adenine mutation at nucleotide position −162 leading to disruption of the −10 promoter sequence. *Cytosine to thymine mutation at nucleotide position 1273 leading to a premature stop codon (TAG). #Insertion of the IS*481* element at nucleotide position 1613 in either the forward (F) or reverse (R) direction. ++Insertion of the IS*481* element at nucleotide position 245 in the reverse (R) direction.

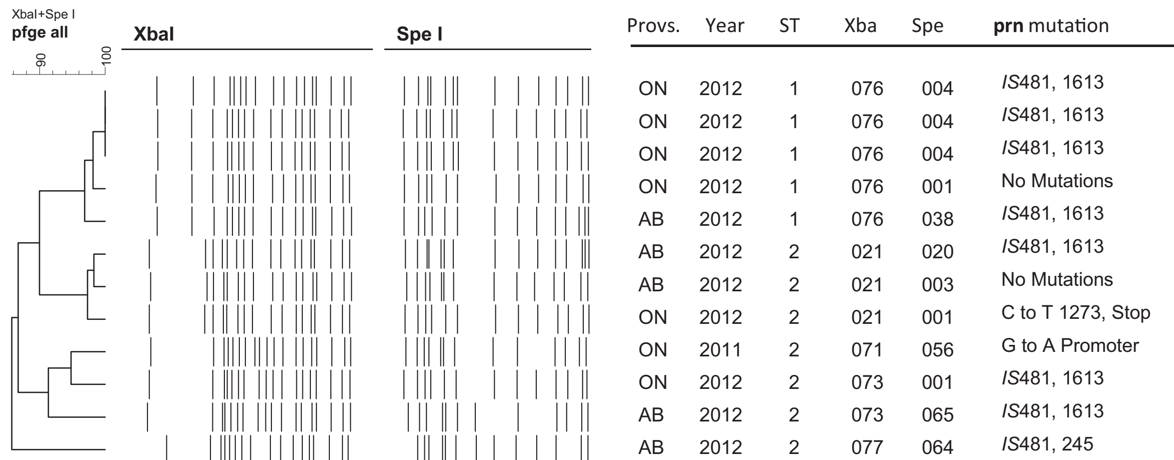

Figure 3. Dendrogram showing relatedness by pulsed-field gel electrophoresis (PFGE) of 12 pertactin-deficient *Bordetella pertussis* isolates in Canada. Isolates are identified by sequence type (ST), *prn* mutation, year, and province (Prov) of isolation (Alberta, AB; Ontario, ON), as well as PFGE profiles generated by restriction enzymes Xba and Spe.

related sequence types (ST-1 and ST-2), characterized by the *fim3B* and *fim3A* genotype, respectively. The PFGE data suggest that the pertactin-negative isolates are not clonal and this observation is also supported by the genetic mutations that we observed in their *prn* genes. The non-clonal nature of the pertactin-negative *B. pertussis* strains has also been observed in Australia.[23] Therefore, our overall data suggest that the pertactin-negative isolates probably arose from multiple independent mutations that occurred recently. It has been hypothesized that these vaccine antigen-negative strains probably developed as a result of vaccine pressure, especially the acellular vaccine types that contain pertactin as one of the few components present as opposed to inactivated whole-cell vaccines. Whether this was related to more intense immunization programs implemented among outbreak communities should be considered in future studies to understand the evolution of *B. pertussis*.

A major limitation of this study was the lack of systematic collection and testing of isolates. This is due to the fact that there is no formal laboratory surveillance program for pertussis in Canada and, therefore, *B. pertussis* isolates are not routinely submitted to the NML for strain characterization. Rather, isolates may be submitted in an ad hoc manner during an outbreak. Even then, bacteriological cultures may not be performed to collect viable organisms for testing, as some jurisdictions now rely solely on PCR to provide laboratory confirmation of pertussis. This inevitably creates an important gap in the critical information necessary to understand the molecular epidemiology of pertussis in Canada and results in our inability to build up a longitudinal database to compare the evolution of strains over time. The importance of maintaining some culture capability to identify and characterize *B. pertussis* strains in order to understand the complexity of how changes in the bacterium may contribute to the overall epidemiology of pertussis has been raised in a recent editorial that appeared in our national communicable disease journal.[17] Another limitation of our study is the fact that there is no standardized system to link vaccination data to cases identified in the country, thereby limiting our ability to know if cases identified are vaccine failures or due to other reasons.

In conclusion, pertactin-negative *B. pertussis* strains have been found in Canada since 2011 and they appeared to be common in two provinces in 2012. This initial study may provide an impetus for the development of a more comprehensive surveillance program for pertussis in Canada to include clinical and epidemiological data, vaccination history, and laboratory characterization of strains.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ijid.2014.08.002.

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