Molecular Epidemiology of the Pertussis Epidemic in Washington State in 2012

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Although pertussis disease is vaccine preventable, Washington State experienced a substantial rise in pertussis incidence beginning in 2011. By June 2012, the reported cases reached 2,520 (37.5 cases per 100,000 residents), a 1,300% increase compared with the same period in 2011. We assessed the molecular epidemiology of this statewide epidemic using 240 isolates collected from case patients reported from 19 of 39 Washington counties during 2012 to 2013. The typing methods included pulsed-field gel electrophoresis (PFGE), multilocus variable number tandem repeat analysis (MLVA), multilocus sequence typing (MLST), and pertactin gene (*prn*) mutational analysis. Using the scheme PFGE-MLVA-MLST-*prn* mutations-Prn deficiency, the 240 isolates comprised 65 distinct typing profiles. Thirty-one PFGE types were found, with the most common types, CDC013 ($n = 51$), CDC237 ($n = 44$), and CDC002 ($n = 42$), accounting for 57% of them. Eleven MLVA types were observed, mainly comprising type 27 ($n = 183$, 76%). Seven MLST types were identified, with the majority of the isolates typing as *prn2-ptxP3-ptxA1-fim3-1* ($n = 157$, 65%). Four different *prn* mutations accounted for the 76% of isolates exhibiting pertactin deficiency. PFGE provided the highest discriminatory power ($D = 0.87$) and was found to be a more powerful typing method than MLVA and MLST combined ($D = 0.67$). This study provides evidence for the continued predominance of MLVA 27 and *prn2-ptxP3-ptxA1* alleles, along with the reemergence of the *fim3-1* allele. Our results indicate that the *Bordetella pertussis* population causing this epidemic was diverse, with a few molecular types predominating. The PFGE, MLVA, and MLST profiles were consistent with the predominate types circulating in the United States and other countries. For *prn*, several mutations were present in multiple molecular types.

O nce a major cause of illness and death among infants and children, with over 160,000 cases annually during the 1920s and 1930s, pertussis, or whooping cough, has become the most frequently reported vaccine-preventable bacterial disease in the United States (1). With the introduction of whole-cell vaccines in the 1940s, the number of reported cases decreased by >99% to a record low in the late 1970s (1–3). Acellular vaccines that contained inactivated pertussis toxin (Ptx), one or more additional bacterial components (i.e., filamentous hemagglutinin [Fha], pertactin [Prn], and fimbriae [Fim] types 2 and 3), and diphtheria and tetanus toxoids were subsequently licensed. These acellular vaccines were recommended for the entire childhood booster series of DTaP (diphtheria, tetanus, and acellular pertussis vaccine) by the end of the 1990s, and in 2005, an additional single dose of diphtheria, tetanus, and acellular pertussis adolescent and adult booster (Tdap) was recommended (4–6). Recently, the Advisory Committee on Immunization Practices (ACIP) expanded booster recommendations to include vaccination of pregnant women to protect mothers and infants (7). Despite these recommendations, pertussis notifications have been steadily increasing since the 1980s, especially among adolescents and adults (8, 9).

During late 2011 and early 2012, several counties in western Washington State observed marked and sustained increases in pertussis over several weeks, prompting the Washington State Department of Health (WA DOH) to declare a statewide epidemic in April 2012. By 16 June, the reported number of cases in 2012 reached 2,520 (37.5 cases per 100,000 residents), a 1,300% increase compared to the same time period in 2011. By the end of the

year, over 4,900 cases were reported, the highest number in 70 years. The incidence was greatest in infants of <1 year and in 10-year-olds and was elevated in 13- and 14-year-olds, the first birth cohort vaccinated solely with acellular vaccines for the childhood series and adolescent booster (10). Because WA DOH school entry data for 2011 to 2012 showed that only 3.5% of students were exempted from pertussis-containing vaccine, suggesting high immunization coverage, and the majority of pediatric cases were reported as vaccinated in this epidemic, it was important to assess how current populations of *Bordetella pertussis* were changing at a molecular level in comparison to the current vaccine (10).

To evaluate the molecular epidemiology of circulating strains of *B. pertussis* in this epidemic, we implemented 3 molecular typing methods that are currently used in the United States and other countries (11–15): pulsed-field gel electrophoresis (PFGE), multilocus variable number tandem repeat analysis (MLVA), and

multilocus sequence typing (MLST). These methods are used to differentiate *B. pertussis* isolates based on whole-genome analysis and sequencing at variable loci throughout the genome (11, 12, 14, 15). Considering that *prn* mutational analysis for this statewide epidemic has been previously described (16), here we combined that *prn* analysis with other molecular typing methods to assess whether those *prn* mutations corresponded to specific molecular types. Our aims were to characterize the molecular epidemiology of the circulating strains of *B. pertussis* during the 2012 Washington State epidemic and for our findings to contribute to the ongoing discussion concerning possible reasons for the reemergence and increased incidence of pertussis in the United States over the past 30 years.

MATERIALS AND METHODS

Bacterial isolate and case reporting. According to the Washington Administrative Code (WAC), health care providers, laboratories, and health care facilities in Washington State are required to report cases of suspected and confirmed pertussis to the local health jurisdiction (LHJ) where the patient resides. In addition, clinical laboratories are also required to submit pertussis isolates to the Washington State Public Health Laboratories (WAPHL). The LHJ performs case investigations and transmits data on case demographics, clinical and laboratory characteristics, and vaccination status to WA DOH using the state's electronic disease reporting surveillance system. Epidemiologists in the WA DOH Office of Communicable Disease Epidemiology (CDE) review these electronic case reports, monitor the reported vaccination status of cases, assign case classifications, transmit data to CDC, and monitor statewide pertussis activity and trends.

Data from surveillance reports and the percentage of cases with isolates submitted to WA PHL suggest that most WA health care providers order pertussis PCR only, reflecting clinical testing trends at the national level. During the study period, the majority of pertussis isolates in WA were obtained at a pediatric academic hospital laboratory, two laboratories serving large hospital systems, and WAPHL.

Two hundred thirty *B. pertussis* isolates from 2012 were forwarded from the WAPHL to the Centers for Disease Control and Prevention (CDC) for molecular characterization, along with 13 isolates submitted in late 2011 and early 2013. Of the 243 isolates sent, 3 were obtained from out-of-state residents and were not included in the analysis. Isolates were cultured on Regan-Lowe agar without cephalexin for 72 h at 35°C.

PFGE. PFGE was conducted using previously described methods (16–18). Colonies were suspended in agarose and formed into plugs for PFGE. Plugs were treated with 20 mg/ml proteinase K for 2 h and then washed several times to remove excess proteinase K. Slices of each plug were digested with XbaI restriction endonuclease for 1.5 h at 37°C. Electrophoresis was performed with a CHEF-Mapper (Bio-Rad Laboratories, Hercules, CA) using the following conditions: gradient, 6 V/cm; included angle, 120°; initial switch time, 2.16 s; final switch time, 35.07 s; ramping factor, linear; temperature, 14°C; run time, 18 h. After electrophoresis, gels were stained with ethidium bromide and DNA bands were visualized with UV light. Tagged image file format (TIFF) images of gels were analyzed, and PFGE profiles were assigned to isolates based on a database of U.S. isolates maintained at CDC, using BioNumerics software version 5.01 (Applied Maths, Austin, TX).

MLVA. MLVA was performed using a duplex reaction targeting variable number tandem repeats (VNTRs) 3 and 4 and a multiplex reaction targeting VNTRs 1, 5, and 6 as previously described (11–13). A HotStarTaq polymerase kit (Qiagen, Valencia, CA) was used to amplify the VNTRs from 60 ng total DNA in each reaction mixture, yielding a final volume of 24 μl (see Table S1 in the supplemental material for primer sequences and concentrations). The amplified products were diluted 1:50 and 1:100 and mixed with 0.5 μl of MapMarker X-rhodamine-labeled 400-bp ladder (BioVentures, Murfreesboro, TN). Sizes were determined

by using the Prism 3130xl genetic analyzer (Applied Biosystems, Foster City, CA); VNTR sizes were determined using GeneMapper version 4.0 software (Applied Biosystems). Amplified product sizes for all strains were compared with those found for *B. pertussis* reference strain Tohama I to determine the repeat count for each locus. The assignment of MLVA type was based on the combination of repeat counts for VNTRs 1, 3a, 3b, 4, 5, and 6 and was consistent with international nomenclature (http://www.mlva.net/).

MLST and *prn* sequence analysis. Our MLST algorithm consisted of 4 DNA targets: fragments of the pertactin gene (*prn*), the first gene in the pertussis toxin operon and its promoter (*ptxA* and *ptxP*), and the fimbrial protein-encoding gene (*fim3*). The primers used for PCR and sequencing are described in Table S1 in the supplemental material. For *prn* typing, PCR was performed using the Expand high-fidelity PCR system (Roche Applied Sciences, Indianapolis, IN). After a 15-min incubation at 95°C, *prn* was amplified, using the primers PRN-AF and PRN-BR, to produce a 1.4-kb PCR product (see Fig. S1 and Table S1) in 30 cycles, each cycle consisting of 30 s at 95°C, 30 s at 55°C, and 2.5 min at 72°C, with a final extension at 72°C for 5 min. For *fim3*, *ptxP*, and *ptxA* typing, PCR amplification was performed using HotStarTaq master mix with the primers indicated in the supplemental material (see Table S1). After a 15-min incubation at 95°C, *ptxP*, *ptxA*, and *fim3* were amplified in 25 cycles consisting of 45 s at 94°C, 45 s at 57°C (*ptxP* and *fim3*) or 58°C (*ptxA*), and a 1-min extension at 72°C. The *ptxP*, *ptxA*, *fim3*, and *prn* sequencing was performed with the primers indicated in Table S1, using the BigDye terminator version 3.1 sequencing kit (Applied Biosystems) as previously described (19). Products were separated on an AB Prism 3130xl genetic analyzer (Applied Biosystems), and sequences were compared to known alleles for each locus. The compositions of these loci were expressed in an allelic profile designated *prn-ptxP-ptxA-fim3*.

Sequencing of *prn* for identification of mutations leading to the lack of pertactin expression was conducted as described previously (16). To identify the G insert at nucleotide 1185 and the stop codon at nucleotide 1273, additional *prn* sequencing was performed with the primers PRN-1627R and PRN-A2F individually for complete coverage of nt 982 to nt 1627 from the PRN-AF and PRN-BR 1.4-kb PCR product (reference to Tohama I) (see Fig. S1 and Table S1 in the supplemental material) (20).

The final molecular typing profile is represented as PFGE-MLVA-MLST-*prn* mutations-Prn deficiency.

Population analysis and index of diversity. The geographic distribution of 2012 cases with and without isolates, by ZIP Code, was analyzed using ArcMap, version 10.1 (ESRI, Redlands, CA). Typing data were compiled using Bionumerics version 5.01. Minimum spanning trees were generated using default settings and either the categorical or binary coefficient, as indicated in the figure legends. The Simpson index of diversity (DI) was calculated as described by Hunter and Gaston (21). The DI is the assessment of the relative frequencies of types defined by a specific molecular typing method (21). In this study, this value indicates the probability of two isolates having different types for each molecular typing method and any combination of typing methods. DI was calculated for MLVA, MLST, PFGE, *prn* mutations, Prn deficiency, and the combinations of all molecular typing methods. All typing data were combined into overarching molecular typing profiles for this study. For example, for the combination PFGE plus MLVA plus MLST, CDC002-MLVA 27-*prn2*-*ptxP3*-*ptxA1*-*fim3-1* was considered a unique type different from CDC002-MLVA 27-*prn2*-*ptxP3*-*ptxA1*-*fim3-2*.

RESULTS

Descriptive characteristics of the epidemic. Of the 4,935 pertussis cases included in this analysis, 4,427 cases were classified as confirmed (84.8% had laboratory confirmation, and 15.2% were epidemiologically linked). Of the 3,602 laboratory-confirmed cases, 90.7% were confirmed by PCR alone, 2.3% by culture alone, and 7.0% by both PCR and culture. Among 2,020 cases aged 3 months to 10 years and 367 cases aged 11 to 12 years, 71.2% and
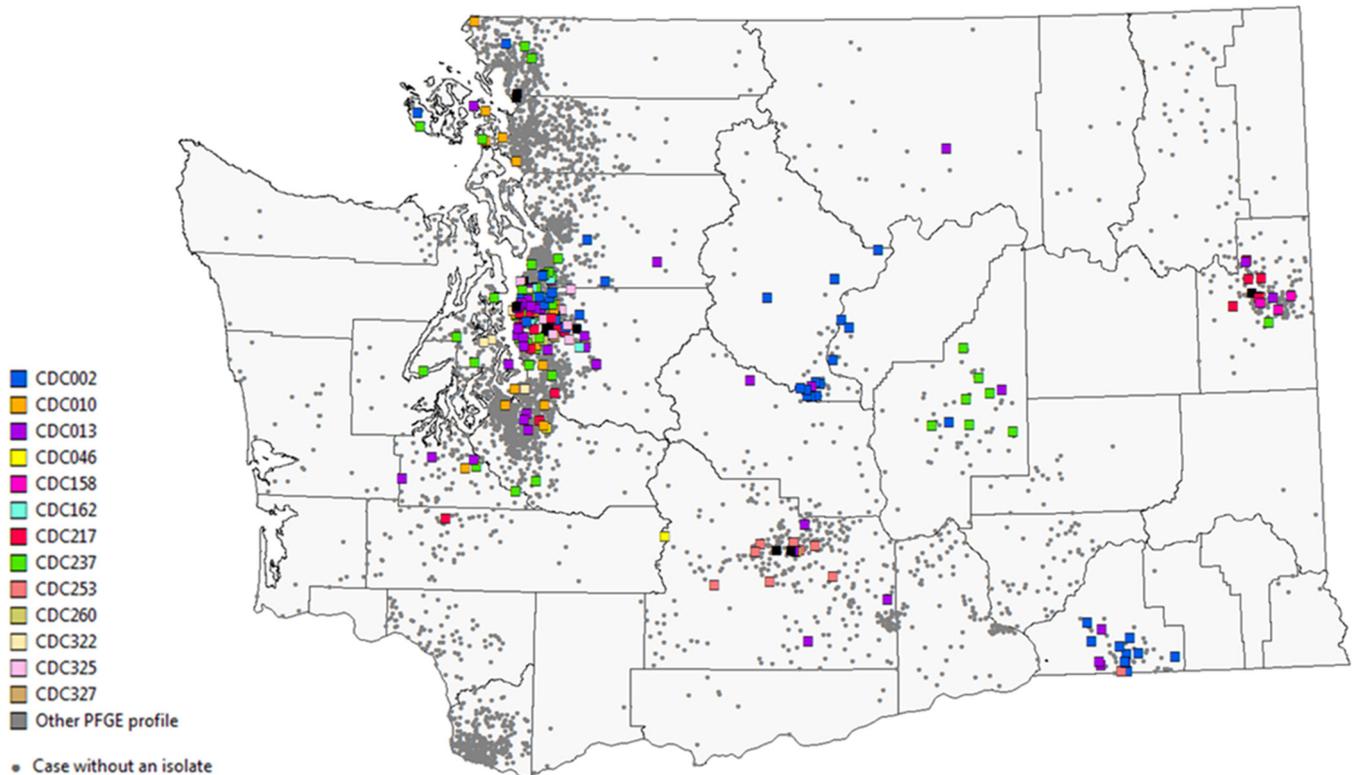
FIG 1 Map of the geographic distribution of cases with and without isolates collected in 2012. Cases were mapped by ZIP Code of residence. Squares represent the locations of case patients with isolates for whom the ZIP Codes and PFGE profiles are known ($n = 233$), with the color corresponding to the PFGE profile of the isolate. Gray circles represent the locations of cases without isolates but with the ZIP Code known ($n = 4,681$). The symbols are placed only within the ZIP Code and do not correspond to the actual location of each case patient's residence.

74.6%, respectively, were up to date for age on their pertussis vaccinations. Among 1,038 adolescents aged 13 to 19 years, 77.5% reported receipt of a Tdap dose. The case patients with isolates (240/4,935) are representative of the overall epidemic case patient population with respect to gender, age, and geographic distribution (Fig. 1 and Table 1). A majority of the isolates were collected during the peak months of the epidemic, April through June of 2012 (Table 1). Cases that were hospitalized were more likely to have had an isolate obtained than cases that were tested in an outpatient setting (37.5% and 18.4%, respectively) (Table 1), which may be consistent with provider practices to conduct PCR testing, as well as more specific testing via culture for infants with severe illness. Differences by race/ethnicity reflect the distribution of the catchment area served by Seattle Children's Hospital Laboratory, which submitted the majority of isolates (59%). Geographic region is defined as counties of case residence east (Eastern Washington) and west (Western Washington) of the Cascade Mountains. Cases residing in Eastern Washington were less likely to have had an isolate obtained than cases in Western Washington, which reflects differences in clinic provider testing practices (e.g., reliance on clinical diagnosis or confirmation by PCR only) between the two regions and the primary catchment area served by Seattle Children's Hospital Laboratory, which is located in Western Washington. However, among the 223 isolates with the case patient's ZIP Code known, the isolates tested were representative of the geographic distribution of the epidemic. Spatial clustering of PFGE types is observed among isolates from Central and Eastern Washington (Fig. 1).

Molecular typing with PFGE, MLST, and MLVA. Sixty-five molecular typing profiles were identified. WA1 and WA2 were the most common profiles, representing 18% and 12% of the total isolates, respectively (Table 2). Forty-two profiles (65%) were represented by a single isolate (Table 2). Thirty-one PFGE types were identified, with the most common types, CDC013 ($n = 51$; 10 profiles), CDC237 ($n = 44$; 2 profiles), and CDC002 ($n = 42$; 6 profiles), accounting for 57% of total isolates (Table 2; Fig. 2). For a majority of PFGE profiles, only a small number of isolates were identified (Table 2; Fig. 2).

Seven MLST types were identified, with the majority of the isolates typing as *prn2-ptxP3-ptxA1-fim3-1* ($n = 157$, 65%; 32 profiles) or *prn2-ptxP3-ptxA1-fim3-2* ($n = 76$, 32%; 27 profiles) (Table 2; Fig. 2 and 3). Isolates with the PFGE type CDC013 were predominately *prn2-ptxP3-ptxA1-fim3-2* ($n = 38$, 75%), while isolates with the PFGE types CDC002 and CDC0237 were predominately *prn2-ptxP3-ptxA1-fim3-1* ($n = 35$, 83%, and $n = 42$, 95%, respectively) (Table 2; Fig. 2).

Eleven MLVA types were observed, mainly comprising MLVA 27 ($n = 183$, 76%; 46 profiles) and MLVA 158 ($n = 37$, 15%; 7 profiles) (Table 2; Fig. 2 and 3). Of the isolates with PFGE types CDC013 and CDC237, 92% and 100%, respectively, were MLVA 27. Seventy-six percent of isolates with the PFGE type CDC002 were MLVA 158 ($n = 32$). Twenty-two of 31 PFGE types were of a single MLVA type.

In comparing MLVA and MLST, we found that a majority of isolates with MLVA 27 and MLVA 158 had the MLST type *prn2-ptxP3-ptxA1-fim3-1* (66% and 84%, respectively) (Table 2; Fig. 3).

TABLE 1 Demographic and clinical characteristics of 2012 Washington State pertussis cases with and without an isolate available for molecular characterization testing

Characteristic	No. (%) without isolate ($n = 4,695$)[a]	No. (%) with isolate tested ($n = 240$)	P value[b]
Gender			
Female	2,529 (53.9)	135 (56.3)	0.470
Male	2,166 (46.1)	105 (43.8)	
Age group (yr)			
<1	359 (7.7)	24 (10.0)	0.523
1–6	909 (19.4)	34 (14.2)	
7–10	972 (20.7)	46 (19.2)	
11–12	378 (8.1)	21 (8.8)	
13–14	559 (11.9)	32 (13.3)	
15–19	543 (11.6)	31 (12.9)	
≥20	975 (20.8)	52 (21.7)	
Race/Ethnicity			
White	2,441 (52.0)	141 (59.0)	0.0002
Hispanic	872 (18.6)	33 (13.8)	
Other	226 (4.8)	23 (9.6)	
Unknown	1,156 (24.6)	43 (17.9)	
Geographic region			
Western WA	3,641 (77.6)	163 (67.9)	0.0005
Eastern WA	1,054 (22.5)	77 (32.1)	
Quarter of onset ($n = 4,918$)	$n = 4,695$	$n = 228$	
January-March	1,110 (23.6)	16 (7.0)	<0.0001
April-June	2,050 (43.7)	127 (55.7)	
July-September	1,077 (22.9)	64 (28.1)	
October-December	458 (9.8)	21 (9.2)	
Hospitalization among infants ($n = 383$)	$n = 359$	$n = 24$	
Yes	66 (18.4)	9 (37.5)	0.022
No	293 (81.6)	15 (62.5)	

[a] One hundred nine cases had a positive culture documented in the case report, but the respective isolates were not submitted to the WAPHL.

[b] χ^2 test for independence was used to determine differences between cases with and without isolates. P values of <0.05 were considered statistically significant.

The 3 isolates typing as WA22 and WA51 were the only isolates observed with the *ptxP1* allele (Table 2; Fig. 2 and 3).

Among pertussis isolates from this statewide epidemic, PFGE alone ($D = 0.87$) accounted for a higher discriminatory power than the combination of MLVA and MLST ($D = 0.67$) (Table 3). The *fim3* locus contributed the most to the discriminatory power of MLST ($D = 0.45$).The combination of PFGE, MLVA, and MLST had an index of diversity of 0.92 (Table 3).

prn mutations. Seventy-six percent ($n = 183$) of the isolates collected during this statewide epidemic were pertactin deficient, as previously described (16). Of the 65 molecular typing profiles identified, only 25 (38%) produced Prn (Table 2). Of the 3 major PFGE profiles, all PFGE type CDC237 isolates ($n = 44$) and 88% of the isolates with PFGE profile CDC002 ($n = 37$) had an IS*481* insertion in the *prn* gene (Table 2). In contrast, 61% of the isolates with PFGE type CDC013 were wild type and produced Prn ($n = 31$). The IS*481* insertion, which can be found in 3 different locations within *prn*, was seen in 35 molecular profiles and associated with five different MLVA types (27, 158, 95, 32, and 5), along with

three MLST types (Table 2; see also Fig. S1 in the supplemental material). The *prn* 22-kb promoter inversion, at nucleotide −74, was present in profiles WA5 and WA17, which corresponded to 84% of isolates with PFGE type CDC010 and 5% of isolates with PFGE type CDC002 (Table 2; see also Fig. S1). Furthermore, the promoter inversion was only seen in the 18 isolates with MLVA 27 and two MLST types that differ by the *fim3* allele (Table 2). The signal sequence (SS) deletion of nucleotides 22 to 109 in *prn* was only seen in two isolates with profile WA22 (Table 2; see also Fig. S1). The *prn* promoter and 5′-end deletion was only seen in the two isolates with profiles WA28 and WA36 (Table 2). These isolates were MLVA 27 and harbored two MLST profiles that differ by the *fim3* allele (Table 2). A point mutation in *prn* resulting in a neutral amino acid change was seen in the isolate with the profile WA32 and did not lead to Prn deficiency (Table 2; see also Fig. S1). Figure 2 shows the clustering of predominately Prn-deficient PFGE types (indicated by red shadows) and predominately Prn-expressing PFGE types (indicated by green shadows).

The combination of PFGE and the *prn* mutations ($D = 0.91$) demonstrated a higher discriminatory power than the combinations of MLVA and the *prn* mutations ($D = 0.70$) and MLST and the *prn* mutations ($D = 0.71$) (Table 3). The combination of all molecular typing data into the scheme PFGE-MLVA-MLST-*prn* mutations-Prn expression accounted for the highest discriminatory power of all combinations of typing methods ($D = 0.94$) (Table 3).

DISCUSSION

We investigated the molecular epidemiology of the largest pertussis epidemic in Washington State in 70 years. Given that the majority of cases were fully vaccinated, we assessed the current population of *B. pertussis* at a molecular level and compared the results to the current vaccine. By PFGE, we found 65 distinct typing profiles that demonstrated high strain diversity with no predominate clone and a large proportion of isolates with pertactin deficiency, and we found a higher discriminatory power of PFGE alone than of MLVA plus MLST. Although it is likely that multiple, possibly interrelated reasons for the recent resurgence of pertussis will ultimately be identified, this study sheds light on the importance of considering bacterial adaption to current vaccines as one possible factor (22–25). This is evident with the surge in strains deficient in pertactin, a known pertussis acellular vaccine immunogen.

The predominate MLVA-MLST type circulating in Washington State was MLVA 27 *prn2-ptxP3-ptxA1-fim3-1*, which aside from the *fim3* allele, coincides with the predominate strain found during the early 1990s to 2009 in a similar study conducted in the United States (11). This strain is currently predominate in Japan, Australia, and parts of Europe (11, 13, 26–28). The molecular profile for 2 strains used to produce the current U.S. vaccine are MLVA 38 *prn1-ptxP1-ptxA2-fim3-1* (Tohama I) and MLVA 167 *prn1-ptxP1-ptxA4-fim3-1* (strain 10536). Few isolates were observed with the *ptxP1* allele, which coincides with the decreased frequency of this allele in circulating strains in the United States, Netherlands, Canada, Sweden, Australia, and Denmark (27–31). MLVA 186 was first seen in the United States in 2007, yet the two isolates with this MLVA type in this epidemic are associated with the MLST profile *prn1-ptxP1-ptxA2-fim3-1* that was observed in the Tohama I strain (11).

Three PFGE profiles predominated within the Washington State epidemic: CDC013, CDC237, and CDC002 (Fig. 1; see also

TABLE 2 Molecular typing profiles of *B. pertussis* (*n* = 240) isolates obtained during the 2012 Washington State epidemic

Profile	PFGE[a]	MLVA[b]	Allele of:				*prn* mutation	Expresses Prn	Total no. of isolates
			prn	*ptxP*	*ptxA*	*fim3*			
WA1	237	27	2	3	1	1	IS*481* insertion[c]	−	42
WA2	2	158	2	3	1	1	IS*481* insertion	−	28
WA3	13	27	2	3	1	2	IS*481* insertion	−	19
WA4	13	27	2	3	1	2	Wild-type *prn*	+	17
WA5	10	27	2	3	1	1	Promoter inversion (−74 nt)	−	16
WA6	253	27	2	3	1	1	IS*481* insertion	−	12
WA7	13	27	2	3	1	1	Wild-type *prn*	+	7
WA8	13	27	2	3	1	1	IS*481* insertion	−	2
WA9	325	27	2	3	1	1	IS*481* insertion	−	7
WA10	217	27	2	3	1	1	Wild-type *prn*	+	6
WA11	217	27	2	3	1	1	IS*481* insertion	−	6
WA12	2	27	2	3	1	1	IS*481* insertion	−	5
WA13	322	95	2	3	1	2	IS*481* insertion	−	5
WA14	2	158	2	3	1	2	IS*481* insertion	−	4
WA15	162	27	2	3	1	2	IS*481* insertion	−	4
WA16	217	32	2	3	1	1	Wild-type *prn*	+	4
WA17	2	27	2	3	1	2	Promoter inversion (−74 nt)	−	2
WA18	2	27	2	3	1	1	Wild-type *prn*	+	2
WA19	46	27	2	3	1	2	IS*481* insertion	−	2
WA20	158	27	2	3	1	2	Wild-type *prn*	+	2
WA21	237	27	2	3	1	2	IS*481* insertion	−	2
WA22	260	186	1	1	2	1	Signal sequence deletion (nt 26 to 109)	−	2
WA23	322	27	2	3	1	2	IS*481* insertion	−	2
WA24	2	27	2	3	1	2	IS*481* insertion	−	1
WA25	10	27	2	3	1	2	IS*481* insertion	−	1
WA26	10	158	2	3	1	1	IS*481* insertion	−	1
WA27	10	158	2	3	1	2	IS*481* insertion	−	1
WA28	13	27	2	3	1	2	Deletion (nt −2090 to −478)	−	1
WA29	13	27	3	3	1	2	IS*481* insertion	−	1
WA30	13	158	2	3	1	1	Wild-type *prn*	+	1
WA31	13	158	9	3	1	2	Wild-type *prn*	+	1
WA32	13	16	2	3	1	2	T at nt 638; neutral amino acid change	+	1
WA33	13	78	2	3	1	1	Wild-type *prn*	+	1
WA34	28	27	2	3	1	1	IS*481* insertion	−	1
WA35	46	27	2	3	1	1	IS*481* insertion	−	1
WA36	46	27	2	3	1	1	Deletion (nt −2090 to 478)	−	1
WA37	46	32	2	3	1	2	Wild-type *prn*	+	1
WA38	82	128	2	3	1	2	Wild-type *prn*	+	1
WA39	140	27	2	3	1	2	Wild-type *prn*	+	1
WA40	158	27	2	3	1	1	Wild-type *prn*	+	1
WA41	162	27	2	3	1	1	IS*481* insertion	−	1
WA42	162	32	2	3	1	2	IS*481* insertion	−	1
WA43	168	27	2	3	1	1	IS*481* insertion	−	1
WA44	177	27	2	3	1	2	Wild-type *prn*	+	1
WA45	217	27	2	3	1	2	Wild-type *prn*	+	1
WA46	217	27	2	3	1	2	IS*481* insertion	−	1
WA47	217	27	2	3	2	1	Wild-type *prn*	+	1
WA48	217	158	2	3	1	1	Wild-type *prn*	+	1
WA49	253	27	2	3	1	2	IS*481* insertion	−	1
WA50	253	5	2	3	1	1	IS*481* insertion	−	1
WA51	258	35	1	1	1	1*[d]	Wild-type *prn*	+	1
WA52	270	27	2	3	1	1	Wild-type *prn*	+	1
WA53	286	27	2	3	1	1	IS*481* insertion	−	1
WA54	295	27	2	3	1	2	IS*481* insertion	−	1
WA55	300	27	2	3	1	1	IS*481* insertion	−	1
WA56	323	27	9	3	1	2	Wild-type *prn*	+	1

TABLE 2 (Continued)

Profile	PFGE[a]	MLVA[b]	prn	ptxP	ptxA	fim3	prn mutation	Expresses Prn	Total no. of isolates
							Allele of:		
WA57	324	200	2	3	1	2	Wild-type prn	+	1
WA58	326	27	2	3	1	1	Wild-type prn	+	1
WA59	327	27	2	3	1	2	Wild-type prn	+	1
WA60	327	128	2	3	1	2	Wild-type prn	+	1
WA61	328	27	2	3	1	1	IS481 insertion	−	1
WA62	330	27	2	3	1	1	IS481 insertion	−	1
WA63	331	27	2	3	1	1	IS481 insertion	−	1
WA64	340	27	2	3	1	1	IS481 insertion	−	1
WA65	347	27	2	3	1	1	IS481 insertion	−	1

[a] PFGE, pulsed-field gel electrophoresis.
[b] MLVA, multilocus variable tandem repeat analysis.
[c] prn::IS481.
[d] fim3-1* has a T at nucleotide 87 instead of a C (4).

Fig. S2 in the supplemental material). CDC013 was the most common PFGE profile in the United States between 2000 and 2009 (40%) but decreased to 11% in 2010 to 2012 (32). The PFGE profile CDC237 was first seen in the United States in 2009 and has rapidly increased to 25% of the 2012 isolates tested (32). Interestingly, 95% of isolates with the profile CDC237 were MLVA 27 and prn2-ptxP3-ptxA1-fim3-1, which are also the predominate MLVA and MLST profiles overall. Unlike the other predominate PFGE
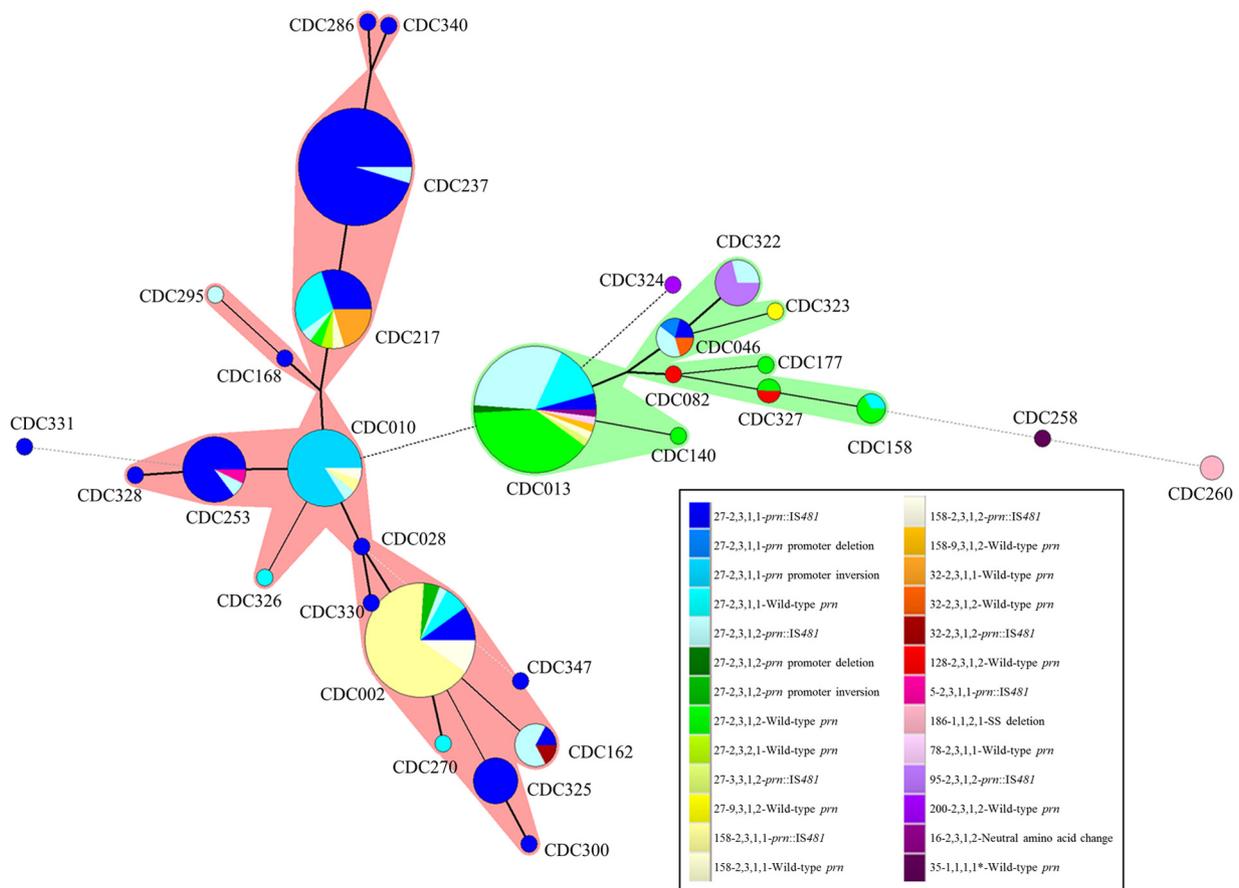

FIG 2 Minimum spanning tree based on PFGE. A binary coefficient was used for clustering. Each circle in the tree represents a different PFGE type, and the type number is indicated within or near the circle. The size of the circle indicates the number of isolates with the particular PFGE type. The lines connecting the circles represent the number of band differences between connecting PFGE types. Heavy solid lines connecting two PFGE types denote types differing by a single band, thin solid lines connect types that differ by two bands, and dotted lines indicate types differing by three or more bands. Each color represents a different MLVA-MLST (prn-ptxP-ptxA-fim3)-prn genotype profile, as indicated in the key. Shadows around circles indicate PFGE types that are closely related based on banding patterns.
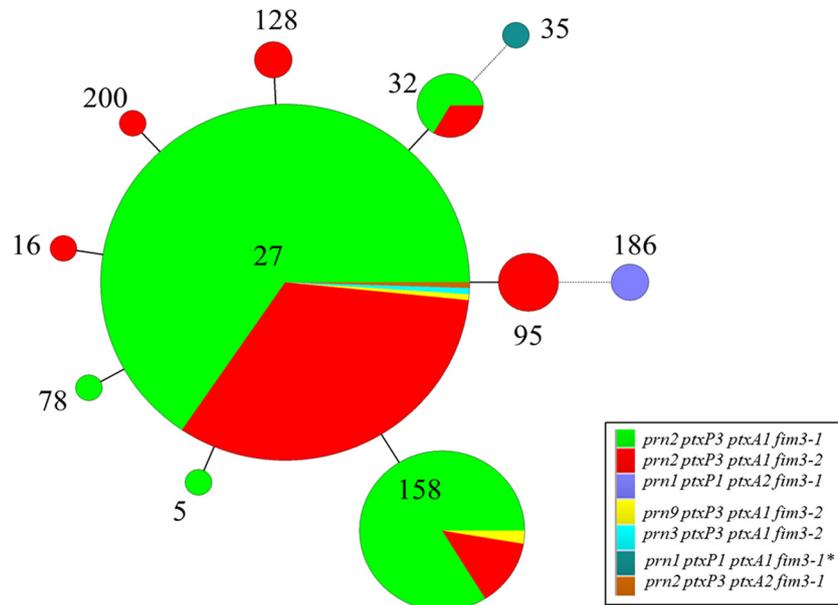
FIG 3 Minimum spanning tree based on MLVA. A categorical coefficient was used for clustering. Each circle in the tree represents a different MLVA type, and the type number is indicated within the circle. The size of the circle indicates the number of isolates with the particular MLVA type. Solid lines connecting circles represent 1 VNTR difference between connecting MLVA types, while dotted lines represent 2 VNTR differences. Each color represents a different MLST type, as indicated in the key.

profiles, CDC002 represented <10% of U.S. isolates from 2000 to 2009 before rapidly increasing to 24% in 2010 to 2012 ([32](#)). Sixty-seven percent of isolates with the profile CDC002 were MLVA 158 and *prn2-ptxP3-ptxA1-fim3-1*.

CDC013 continues to have a majority of isolates with the *fim3-2* allele, while the majority of the CDC237 and CDC002 isolates possess the *fim3-1* allele ([Fig. 2](#)). Until 2009, increased pertussis case reporting was correlated with the emergence of *fim3-2* (designated *fim3B* in reference 11) in the United States. That is not the case for this epidemic, where the reemergence of the *fim3-1* allele (designated *fim3A* in reference 11) was seen. The *fim3-1* allele has not predominated in U.S. isolates since the early 2000s ([11](#)).

By comparing the index of diversity values of the molecular

TABLE 3 Simpson's diversity indices for typing strategies

Typing method[a]	Type count	Index of diversity
PFGE	31	0.87
MLVA	11	0.40
MLST	7	0.47
PFGE + MLST	43	0.90
PFGE + MLVA	43	0.91
MLVA + MLST	18	0.67
PFGE + MLVA + MLST	57	0.92
prn mutations	6	0.51
PFGE + *prn* mutations	40	0.91
MLVA + *prn* mutations	16	0.70
MLST + *prn* mutations	14	0.71
Prn deficiency	2	0.36
PFGE + MLVA + MLST + *prn* mutations + Prn deficiency	65	0.94

[a] PFGE, pulsed-field gel electrophoresis; MLVA, multilocus variable number tandem repeat analysis; MLST, multilocus sequence typing; *prn*, pertactin gene.

typing methods implemented in this study, it was evident that different methods demonstrated very different snapshots of diversity. Similar to what is seen in the United States as a whole, as well as in other countries, this epidemic does not demonstrate the presence of a large number of alleles for the *prn*, *ptxP*, and *ptxA* genes, indicating a low level of diversity based on these MLST loci ([11](#), [26](#), [29](#)). The *fim3* locus contributed most to the diversity of the MLST profiles. Our finding of a higher discriminatory power for PFGE than for the combination of MLVA and MLST was also seen in a molecular typing study conducted on Swedish isolates ([15](#)). Seeing that PFGE is a depiction of diversity on a genomic scale provides evidence that these isolates are changing more rapidly on a genomic level than within the individual genes and regions targeted in MLVA and MLST. With the indication that diversity is occurring at a genomic level, as seen by PFGE and *prn* mutations (specifically the IS*481* insertion), it is important to address the need for a whole-genome analysis to make more definitive conclusions about correlation. With the lack of a unified typing scheme and the diversity seen in this one epidemic, it has become increasingly necessary to develop a universally applicable format for reporting the molecular epidemiology of *B. pertussis* populations that is consistent with the guidelines for bacterial genetic nomenclature. This will allow for more efficient comparisons across multiple epidemics in multiple countries.

Though it was first identified in an isolate from 1994, it was not until 2010 that pertactin deficiency began steadily increasing in *B. pertussis* isolates in the United States ([16](#), [33](#)). To date, the United States has seen a significantly higher number of pertactin-deficient *B. pertussis* strains than other countries, such as France in 2007 (7%), Japan from 1990 to 2009 (27%), or Finland from 2006 to 2011 (2%) ([34–36](#)). The proportion of pertactin-deficient isolates has begun to steadily increase in other countries, including France, increasing from 2% in 2005 to 14% in 2012, which is

similar to the progressive increases seen in the United States and Australia (16, 37–39). A large proportion of isolates (76%) in this study were pertactin deficient due to one of four different mutations previously identified in the *prn* gene (16, 20, 34–37). The presence of the SS deletion in the 2 isolates with PFGE profile CDC260, MLVA 186, and MLST profile *prn1-ptxP1-ptxA2-fim3-1* does not correlate with what was seen in Japan from 1990 to 2009, where the *prn1* SS deletion predominated among pertactin-deficient isolates (73%) and was seen in 3 different MLVA types (MLVA 186, MLVA 194, and MLVA 226) (35). The *prn1* SS deletion was first seen in Japan in 2000 and has shown more MLVA diversity than was seen in the Washington State epidemic (35). One isolate (collected during 2007) out of 600 in the United States had MLVA type 186 and had the same PFGE and MLST profiles as seen with the 2 isolates from the Washington State epidemic (11). The only two pertactin-deficient isolates collected in Finland from 2006 to 2011 also harbored the *prn1* SS deletion (36). It should be noted that while IS481 insertions are seen throughout multiple MLVA types and MLST profiles, the additional three mutations loosely associate with two MLVA types and two MLST profiles, which may be due in part to the low number of isolates collected in this study (Table 2).

Since the majority of cases in this statewide epidemic occurred among school-aged children that were up to date for their age for pertussis-containing vaccine as recommended by the ACIP, it is important to consider what factors may have led to this surge in reported cases in Washington State, as well as in the United States as a whole. One current hypothesis centers on the idea that *B. pertussis* strains harboring *prn* mutations (i.e., escape mutants) possibly have a selective advantage in individuals vaccinated with acellular vaccines (40, 41). As previously mentioned, the majority of strains circulating in the Washington State epidemic are divergent from the two current U.S. vaccine strains, which both express *prn1*. A study conducted in Italy indicated that the *prn* vaccine allele (*prn1*, which encodes the Prn1 antigenic variant) was identified at a lower frequency in vaccinated individuals and was seen in an increased number of isolates collected from areas of low vaccination (40). *prn1* and *prn2* differ by the presence of 5 amino acid repeats in variant region 1 of *prn* but are recognized equally efficiently by the T-cell response in mice and humans (42). Here, we see *prn2* predominating in a highly vaccinated population, which would provide evidence in support of this hypothesis. Epidemiological analysis suggests that vaccinated persons have increased susceptibility to pertactin-deficient strains compared to their susceptibility to strains expressing pertactin (40, 41). There has been no direct evidence that pertactin-deficient strains are more virulent in humans than strains expressing pertactin (38, 40). Through our analysis, it is clear that the currently circulating strains are diverse on a genomic level. The rapidly increasing availability of whole-genome sequencing and analyses, specifically through analysis of additional virulence-related genes, will allow direct investigation of this hypothesis by determining whether pertactin is a major virulence-related gene affected by mutations and will likewise provide answers with regard to what genes may provide these strains associated with pertussis disease with a selective advantage in vaccinated populations.

In an era of increasing provider reliance on pertussis PCR for rapid diagnosis and treatment of suspected cases, this study demonstrates the importance of maintaining culture testing practices. With the highest number of pertussis cases reported in 2012 in the United States since the 1950s, understanding the molecular epidemiology of currently circulating strains of *B. pertussis* is critical. Providers should be made aware that isolation of *B. pertussis* is necessary so that genetic characterization and analysis can be conducted to monitor trends in strain circulation. This evaluation of isolates from a large pertussis epidemic in Washington State has contributed further to our understanding of strain evolution in the United States and suggests that as current molecular typing techniques advance, our understanding of the *B. pertussis* diversity seen on a genomic level will continue to evolve.

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