Bordetella pertussis Clones Identified by Multilocus Variable-Number Tandem-Repeat Analysis

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Multilocus variable-number tandem-repeat analysis (MLVA) of 316 *Bordetella pertussis* isolates collected over 40 years from Australia and 3 other continents identified 66 MLVA types (MTs), including 6 predominant MTs. Typing of genes encoding acellular vaccine antigens showed changes that may be vaccine driven in 2 MTs prevalent in Australia.

Despite longstanding vaccination programs, pertussis remains endemic to many industrialized countries, including Australia, Canada, Italy, Japan, the Netherlands, Switzerland, and the United States, all of which have reported recent increases in incidence (*1*). Although pertussis is classically a disease of infants and children, this increase has been mainly among adults and adolescents (*2,3*). Factors contributing to pertussis resurgence remain unclear, but possible causes are waning immunity, suboptimal vaccine coverage, improved surveillance and diagnosis, the switch from whole cell vaccine (WCV) to acellular vaccine (ACV), and adaptation of circulating *Bordetella pertussis* strains (*4–9*). To determine the global epidemiology of pertussis, we analyzed an international collection of *B. pertussis* isolates collected mainly over the past 40 years.

The Study

We used 8 variable-number tandem-repeats, including 6 from Schouls et al. (*10*), to develop a multiplex PCR multilocus variable-number tandem-repeats analysis (MLVA)

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assay (Table 1; online Technical Appendix, www.cdc.gov/EID/content/16/2/297-Techapp.pdf) and used it to characterize 316 *B. pertussis* isolates from 12 countries on 4 continents, including 208 isolates from Australia and 87 isolates representative of common pulsed field gel electrophoresis types from Canada, Japan, Finland, and the United States (complete list available from authors). The Simpson index of diversity (*D*) ranged from 0.02 to 0.73 per locus with a combined *D* of 0.911 (Table 2). The isolates were resolved into 66 MLVA types (MTs) (complete list available from authors). Thirty-five MTs were represented by single isolates, including 15 of 208 isolates from Australia and 10 of 49 isolates from Japan. Thirty-seven MTs were previously found in Europe (*10,11*) and 27 were novel. Fourteen MTs were found in ≥2 countries or regions.

The 208 isolates from Australia were grouped into 37 MTs, of which the 4 most prevalent represented 65.4% of the isolates: MT27, 13.5%, including 1 isolate from 1973 and the others from the 1990s to 2008; MT29, 21.6%, observed since 1972; MT70, 21.2%, 1996–2005, mostly since ACV introduction in 1997; and MT64, 9.1%, during 1989–2002. Prevalence trends of the 4 most common MTs were analyzed for 3 periods determined by vaccine type(s) in use: WCV (prior to 1997), the transition period of both WCV and ACV (1997–1999), and ACV only (2000 onward) (Figure 1). MT64 prevalence was steady over time. MT29 decreased while MT27 and MT70 increased. Trends in Australia for MT27 and MT29 were similar to those observed in the United Kingdom (*11*) and the Netherlands (*10*). D values were 0.86, 0.83, and 0.83 for WCV, transition, and ACV periods, respectively. This slight decrease in genetic diversity might indicate expansion of clones that are better adapted to ACV-induced immunity.

We typed 5 genes, the products of which are used in ACVs (*prn, ptxA, fim2, fim3,* and *fhaB*), using the method of Chan et al. (*12*) to assess the effect of the switch from WCV to ACV on prevalence of the 4 predominant MTs (MT27, MT29, MT64, and MT70) in Australia. Isolates from these MTs have the same *ptxA1* and *fhaB1* alleles but vary in the other 3 genes investigated (complete list available from authors). The predominant ACV used in Australia is from GlaxoSmithKline (GSK) (Research Triangle Park, NC, USA), which contains pertussis toxoid, filamentous hemagglutinin, and pertactin but no fimbriae (FIMs). The strain used for GSK ACV contains the alleles *prn1, ptxA2,* and *fhaB1* (*10,13*). However, ACV from Sanofi-Aventis (Pasteur, Lyon, France), which contains FIM2+3 in addition to pertussis toxoid, pertactin, and filamentous hemagglutinin with unknown allele types, is also licensed in Australia, complicating interpretation of variation in *fim* genes. On the basis of their frequencies and late appearance, *fim2-2* and *fim3-B* are not likely to be the vaccine alleles. A significant increase (p<0.005) of *prn2* (36% vs. 3%)*, fim2-2* (34% vs.

Table 1. Primers used in study of *Bordetella pertussis* clones Identified by multilocus variable-number tandem-repeat analysis

Primer name	Sequence, 5′ → 3′	Genome coordinates*	Mix	Concentration,† μM	Reference
BP-VNTR1-DF	VIC-CCTGGCGGCGGGAGACGTGGTGGTG	2194507	1	0.13	(*10*)
BP-VNTR1-DR	AAAATTGCGGCATGTGGGCTGACTCTGA	2194862	1		(*10*)
BP-VNTR2-BF	VIC-CGCGCCGCCTACGACCGCTATGG	2647550	2	0.08	(*10*)
BP-VNTR2-BR	CCCGCGCCGAAGATCTCGCCAAAGATAT	2647412	2		(*10*)
BP-VNTR3-BF	FAM-GCCTCGGCGAAATTGCTGAAC	2591464	2	0.23	(*10*)
BP-VNTR3-BR	GCGGGCGAGGAAACGCCCGAGACC	2591350	2		(*10*)
BP-VNTR4-CF	NED-CGTGCCCTGCGCCTGGACCTG	185211	2	0.08	(*10*)
BP-VNTR4-BR	GCCGCTGCTCGACGCCAGGGACAA	185000	2		(*10*)
BP-VNTR5-BF	PET-GAAGCCGGCCCACCCGAGCTCCAGGCTCTT	1005290	1	0.06	(*10*)
BP-VNTR5-BR	TGCCGGGTTTCGGCATCTCGATGGGATACG	1005177	1		(*10*)
BP-VNTR6-EF	FAM-CCAACGGCGGTCTGCTGGGTGGTC	2099525	1	0.06	(*10*)
BP-VNTR6-FR	CGCCGCCCGCTGCGCCGCTACC	2099315	1		(*10*)
VNTR7F2	PET-ATCAGGAAACCCACCACCACGCCGG	124402	2	0.08	This study
VNTR7R2	GTCACCAGCCCGCAGTACTGGCG	124585	2		This study
VNTR8F2	NED-TGGGTGTCTCCGTGATAGTGAGCACTTACAC	444776	1	0.19	This study
VNTR8R2	CTGGCGCAAAAACAGTAAGCCCGCACG	444981	1		This study

*Based on genome sequence position of the Tohama I strain; VNTR, variable-number tandem-repeat.
†Concentrations listed are for forward and reverse primers separately.

8%), and *fim3-B* (24% vs. 0%) was observed in the ACV period in comparison to the WCV period.

This increase of allelic frequency is better reflected in changes in antigenic profiles. MT27 has 3 profiles (*prn1, fim2*-1, *fim3*-A*; prn2, fim2*-1, *fim3*-A*;* and *prn2, fim2*-1, *fim3*-B). The first profile was seen once in the WCV period, whereas the other 2 first appeared in the WCV/ACV transition period and increased in frequency in the ACV period; the third profile, which differed by 2 alleles from the first, was more frequent. The appearance of *prn2* in the second profile and additional change from *fim3-A* to *fim3-B* in the third represent increases in prevalence of alleles absent from ACV. MT29 also has 3 profiles ([*prn1, prn2,* or *prn3*], *fim2*-1, *fim3*-A), which differ in *prn* only. Most MT29 isolates carry *prn3,* and the profile is prevalent in both WCV and ACV periods, with no obvious increase in non-ACV alleles.

MT70 and MT64 both have uniform allelic profiles (*prn1*, *fim2*-2, *fim3*-A and *prn1*, *fim2*-1, *fim3*-A, respectively)**.** However, MT70 (with *fim2-2,* not likely to be in ACV) increased significantly over the study period while MT64 with all alleles likely to be in ACV remained steady.

Overall, the frequency of MT27 and MT70, with non-ACV alleles, increased significantly (p<0.0001) and correlated with the introduction of ACV, suggesting that antigenic changes could be driven by selection pressure.

The 2 MTs predominant in Australia were also prevalent in other countries and possibly have a global distribution. MT27 (18% of isolates) was found in 8 countries and MT29 (17% isolates) in 5. However, absence of an MT in a country might result from the small samples used**.** MT27 and MT29 were the most common types in the Netherlands (*10*) and the United Kingdom (*11*). MT10, MT64, MT70, MT84, and MT186 were also relatively common. MT10 and MT186 were found predominantly in Japan, although each had been found elsewhere, in China (1957) and Hong Kong (2002), respectively. MT64 was predominantly from Australia with 1 isolate from Japan, and MT70 was only found in Australia. However, all of these frequent MTs (except MT186) have been observed before. MT10 was frequent in the United Kingdom in the prepertussis vaccine era, while MT70 was common during 1998–2001 (*11*).

Nine isolates, including Tohama I, indentified in samples of pertussis strains collected during the 1920s–1950s

Table 2. Diversity of variable-number tandem-repeat analysis loci *Bordetella pertussis* isolates*

Locus	No. repeats	Global (this study)		Australia (this study)		The Netherlands (*10*)	
		No. alleles	D	No. alleles	D	No. alleles	D
VNTR1	2–12	6	0.58	4	0.63	7	0.26
VNTR2	2–5	4	0.02	2	0.01	3	N/A
VNTR3a	2–8	4	0.40	4	0.43	10	0.18
VNTR3b	0–10	5	0.21	5	0.21	4	0.15
VNTR4	2–9	7	0.34	5	0.24	8	0.21
VNTR5	3–9	6	0.20	4	0.19	7	0.18
VNTR6	2–11	8	0.72	5	0.70	8	0.60
VNTR7	3–4	2	0.01	1	0.00	NA	NA
VNTR8	2–4	3	0.16	2	0.03	NA	NA

*D, Simpson index of diversity; VNTR, variable-number tandem-repeat; NA, not applicable.
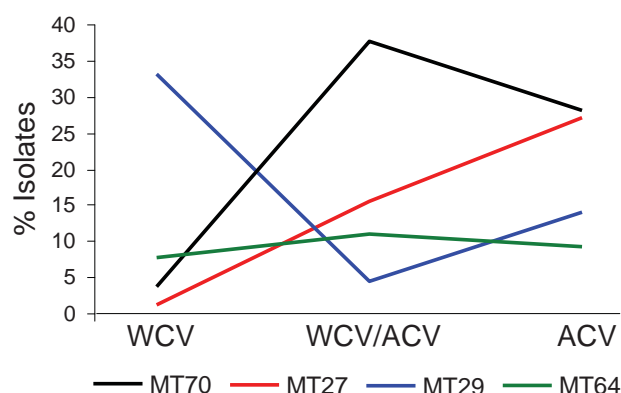
Figure 1. Temporal trends of predominant multilocus variable-number tandem-repeat analysis (MLVA) types in Australia. Isolates of 4 major MLVA types (MT70, MT27, MT29, and MT64) obtained in Australia were divided into 3 periods: whole cell vaccine (WCV) (before 1997), transition from WCV to acellular vaccine (ACV) (1997–1999) and ACV (2000 onward).

from 5 countries (China, France, Japan, United Kingdom, and United States) were distributed among 7 MTs: MT10, MT12, MT75, MT83, MT127, MT205, and MT206, 2 of which were also represented among recent strains: MT10, 6 isolates from Japan 1989–2007; and MT75, 1 isolate from France in 1993. The remaining 5 MTs were either unique or shared only among the 9 early isolates.

MLVA data were used to construct a minimum spanning tree (MST) (Figure 2). The 66 MTs were grouped into 2 clonal complexes and 9 singletons. Most MTs (54 of 66) belong to 1 clonal complex and 3 (MT186, MT187, and MT194) belong to another. Relationships between singletons with multiple allelic differences are not robust because they can be connected to other nodes equally. Thus, the MST cannot be rooted to infer the direction of change. Two internationally predominant MTs (27 and 29) are closely related with 1 allele difference. MT10, prevalent in Japan, is also closely related to MT29, with 1 allele difference. MT29, first isolated in the prevaccine era in the United Kingdom (*11*), has the highest number of SLVs and was found over 4 continents, which suggests that it arose early. Because MT10 and MT27 have a high frequency of SLVs, both likely emerged quite early. MT10 was isolated as early as 1957 in China and MT27 in 1950 in the Netherlands (*10*). Two high-frequency MTs (MT64 and MT70) were found in Australia only recently, with few SLVs, and may have contributed to the resurgence of pertussis in Australia.

Conclusions

Analysis of 208 isolates from Australia and representative isolates of common pulsed-field gel electrophoresis types from Canada, Japan, Finland, and the United

States identified 6 predominant MTs (clones). Two (MT27 and MT29) were distributed worldwide, while 4 (MT10, MT64, MT70 and MT186) predominated in specific countries. Several MTs have persisted over long periods, including 3 that have circulated for at least half a century. Typing of genes encoding ACV antigens showed that use of ACV may have driven antigenic changes of 2 MTs now predominant in Australia.
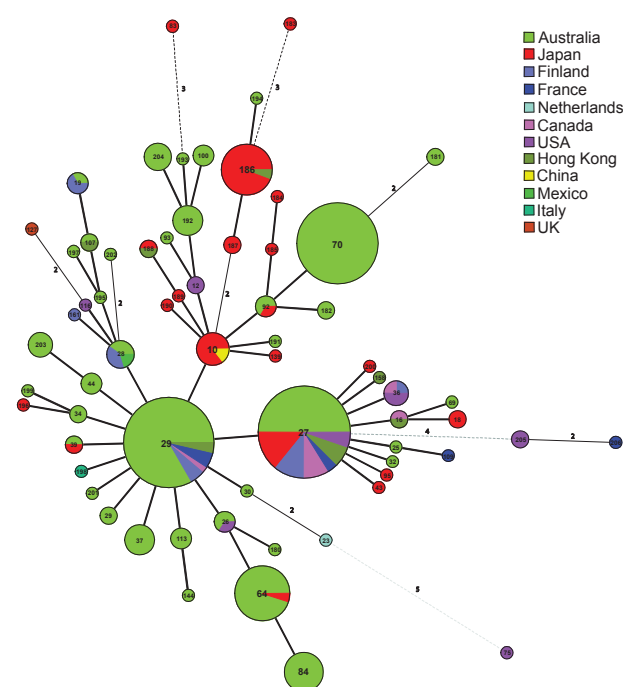
Figure 2. Minimum spanning tree (MST) of multilocus variable-number tandem-repeat analysis (MLVA types for global *Bordetella pertussis* isolates. The MST produced in Bionumerics (Applied Maths, Kortrijk, Belgium) used categorical coefficient and the eBURST priority rule of the highest number of single-locus changes for the clustering. Each circle represents an MLVA type with the type number in the circle. Thick lines, types differing by a single MLVA locus; thin lines, double-locus variants; dotted lines, 2 types differing by >2 MLVA loci. The size of the circle reflects the number of isolates with a given MLVA type. The color codes for country of origin are shown, and pie charts within a circle are used to indicate the proportion of isolates.

Mr Kurniawan is a PhD student in medical microbiology at the University of New South Wales, Sydney, Australia. His research interests include molecular epidemiology and evolution of *B. pertussis.*

References

1. Tan T, Trindade E, Skowronski D. Epidemiology of pertussis. Pediatr Infect Dis J. 2005;24:S10–8. DOI: 10.1097/01. inf.0000160708.43944.99
2. Guris D, Strebel PM, Bardenheier B, Brennan M, Tachdjian R, Finch E, et al. Changing epidemiology of pertussis in the United States: increasing reported incidence among adolescents and adults, 1990–1996. Clin Infect Dis. 1999;28:1230–7. DOI: 10.1086/514776
3. Celentano LPMDM, Massari MD, Paramatti DD, Salmaso SD, Tozzi AE, EUVAC-NET Group. Resurgence of pertussis in Europe. Pediatr Infect Dis J. 2005;24:761–5. DOI: 10.1097/01. inf.0000177282.53500.77
4. Poynten M, McIntyre PB, Mooi FR, Heuvelman KJ, Gilbert GL. Temporal trends in circulating *Bordetella pertussis* strains in Australia. Epidemiol Infect. 2004;132:185–93. DOI: 10.1017/S095026880300164X
5. Bamberger ES, Srugo I. What is new in pertussis? Eur J Pediatr. 2008;167:133–9. DOI: 10.1007/s00431-007-0548-2
6. Mooi FR, van Loo IH, King AJ. Adaptation of *Bordetella pertussis* to vaccination: a cause for its reemergence? Emerg Infect Dis. 2001;7:526–8. DOI: 10.3201/eid0703.010308
7. Mooi FR, He Q, Guiso N. Phylogeny, evolution, and epidemiology of *Bordetellae*. In: Locht C, editor. *Bordetella* molecular microbiology. Wymondham (UK): Horizon Scientific Press; 2007. p. 17–45.
8. Kodama A, Kamachi K, Horiuchi Y, Konda T, Arakawa Y. Antigenic divergence suggested by correlation between antigenic variation and pulsed-field gel electrophoresis profiles of *Bordetella pertussis* isolates in Japan. J Clin Microbiol. 2004;42:5453–7. DOI: 10.1128/JCM.42.12.5453-5457.2004
9. Byrne S, Slack AT. Analysis of *Bordetella pertussis* pertactin and pertussis toxin types from Queensland, Australia, 1999–2003. BMC Infect Dis. 2006;6:53. DOI: 10.1186/1471-2334-6-53
10. Schouls LM, van der Heide HG, Vauterin L, Vauterin P, Mooi FR. Multiple-locus variable-number tandem repeat analysis of Dutch *Bordetella pertussis* strains reveals rapid genetic changes with clonal expansion during the late 1990s. J Bacteriol. 2004;186:5496–505. DOI: 10.1128/JB.186.16.5496-5505.2004
11. Litt DJ, Neal SE, Fry NK. Changes in genetic diversity of the *Bordetella pertussis* population in the United Kingdom between 1920 and 2006 reflect vaccination coverage and emergence of a single dominant clonal type. J Clin Microbiol. 2009;47:680–8. DOI: 10.1128/JCM.01838-08
12. Chan WF, Maharjan RP, Reeves PR, Sintchenko V, Gilbert GL, Lan R. Rapid and accurate typing of *Bordetella pertussis* targeting genes encoding acellular vaccine antigens using real time PCR and high resolution melt analysis. J Microbiol Methods. 2009;77:326–9. DOI: 10.1016/j.mimet.2009.03.007
13. van Amersfoorth SC, Schouls LM, van der Heide HG, Advani A, Hallander HO, Bondeson K, et al. Analysis of *Bordetella pertussis* populations in European countries with different vaccination policies. J Clin Microbiol. 2005;43:2837–43. DOI: 10.1128/JCM.43.6.2837-2843.2005

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Technical Appendix

Isolates Used in This Study

A complete list of the isolates used in this study is available from the author. The majority were recent isolates: 208 from Australia (1970s–2008), 49 from Japan (1989–2007), excluding the Tohama I strain, with 36 being representatives from the 48 pulsed field gel electrophoresis types identified previously by Kodama et al. (*1*), 15 from Finland (1991–2006), 11 from Hong Kong (2002–2006), 8 from France (1993– 2007), 12 from the United States (2002–2006), 8 from Canada (1994–2005) and 1 each from the Netherlands (1992), Italy (1994), China (1957), United Kingdom (1920), and Mexico (year of isolation unknown). The isolates from Canada, Finland, and the United States were representatives of most frequent pulsed field gel electrophoresis types from the respective countries and therefore represent the prevalent strains currently circulating in these countries. Nine strains, including Tohama I, were isolated before 1960, one each from China, France, Japan, and the United Kingdom, and 5 from the United States.

Multilocus Variable-Number Tandem Repeat Analysis

We explored new variable-number tandem repeat (VNTR) analysis loci in addition to the 6 (VNTRs 1–6) previously identified by Schouls et al. (*2*). Forty-one potential VNTR loci from the *B. pertussis* Tohama I genome (*3*) were identified and screened on a panel of 18 *B. pertussis* isolates selected on the time and locality of isolation (Supplementary Table 1). Two new VNTR loci named VNTR7 and VNTR8 were found to be variable in the 18 isolates tested with 2 and 3 alleles, respectively. They were added to the initial set of 6 to develop an 8-plex PCR reaction. Because no conditions satisfied the simultaneous amplification of all 8 products consistently, the primers were divided between two 4-plex PCR assays with VNTR 1, 5, 6, and 8 in 1 reaction and VNTR 2, 3, 4, and 7 in the other. The thermal cycling parameters for the 2 assays were identical, except that the annealing temperature was 68°C for the first and 60°C for the second 4-plex PCR.

The primers and dye labels are listed in Table 1 in the main text. Each 4-plex PCR was performed in 20-μL volumes (100 μM dNTPs, 20 mM Tris-HCl, 10 mM $(NH_4)_2SO_4$, 10 mM KCl, 2 mM $MgSO_4$, 0.1% Triton X-100, 0.25 units/μL Taq DNA polymerase [New England Biolabs, Ipswich, MA, USA]). The thermal cycling parameters were an initial denaturation step at 96°C for 3 min, followed by 40 cycles of 96°C for 20 s, 68°C for 30 s for mix 1 or 60°C for 30 s for mix 2, 72°C for 1 min and 30 s, and a final extension step of 72°C for 20 min. Final PCR products for mix 1 and mix 2 were diluted 1:100 and 1:200, respectively before the fragments were separated on an ABI 3700 DNA sequencer. All VNTRs were amplified consistently.

For each VNTR, the size of the PCR product was converted to number of repeat units as alleles. For consistency, we used the same MLVA type numbering scheme as for the 5-VNTR loci (excluding VNTRs 2, 7, and 8) (2). When a 5-locus type is subdivided, because of differences at one or more of the 3 additional loci, the most frequent subtype takes the type number and any other subtypes were named by adding a letter to the type number. Only 1 MLVA type has a subtype in the 8-locus scheme. MLVA type 29b (2 isolates, profile 8-3-7-0-7-6-10-3-3) is a subtype of MLVA type 29 (profile 8-3-7-0-7-6-9-3-3). Therefore, MLVA types based on 5 VNTR loci are compatible with those based on 8 loci, which allows comparison with previous typing data. Although the extra 3 VNTRs do not contribute greatly to discriminatory power, their inclusion is useful because they act as confirmatory markers for some genotype divisions. MLVA type is abbreviated as MT in the main text.

Bioinformatics

A minimum spanning tree (MST) was constructed by using Bionumerics (Applied Maths, Kortrijk, Belgium) based on categorical coefficient and the eBURST (4) priority rule of the highest number of single-locus changes for the clustering. A clonal complex was defined as consisting of isolates that were single locus variants (SLVs) of the predominant MLVA type (4). The Simpson's index of diversity (D value) was calculated by using an in-house program, the MLEECOMP package (5).

References

1. Kodama A, Kamachi K, Horiuchi Y, Konda T, Arakawa Y. Antigenic divergence suggested by correlation between antigenic variation and pulsed-field gel electrophoresis profiles of *Bordetella*

pertussis isolates in Japan. J Clin Microbiol. 2004;42:5453–7. PubMed DOI: 10.1128/JCM.42.12.5453-5457.2004

2. Schouls LM, van der Heide HG, Vauterin L, Vauterin P, Mooi FR. Multiple-locus variable-number tandem repeat analysis of Dutch *Bordetella pertussis* strains reveals rapid genetic changes with clonal expansion during the late 1990s. J Bacteriol. 2004;186:5496–505. PubMed DOI: 10.1128/JB.186.16.5496-5505.2004

3. Parkhill J, Sebaihia M, Preston A, Murphy LD, Thomson N, Harris DE, et al. Comparative analysis of the genome sequences of *Bordetella pertussis, Bordetella parapertussis* and *Bordetella bronchiseptica.* Nat Genet. 2003;35:32–40. PubMed DOI: 10.1038/ng1227

4. Feil EJ, Li BC, Aanensen DM, Hanage WP, Spratt BG. eBURST: inferring patterns of evolutionary descent among clusters of related bacterial genotypes from multilocus sequence typing data. J Bacteriol. 2004;186:1518–30. PubMed DOI: 10.1128/JB.186.5.1518-1530.2004

5. Pupo GM, Lan R, Reeves PR, Baverstock P. Population Genetics of *Escherichia coli* in a Natural Population of Native Australian Rats. Environ Microbiol. 2000;2:594–610. PubMed DOI: 10.1046/j.1462-2920.2000.00142.x