

# Newly Emerging Clones of *Bordetella pertussis* Carrying *prn2* and *ptxP3* Alleles Implicated in Australian Pertussis Epidemic in 2008–2010

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Australia is experiencing a prolonged epidemic of pertussis that began in 2008. A total of 194 *Bordetella pertussis* isolates collected from 2008 through 2010 were typed by single-nucleotide polymorphism (SNP) analysis, by multilocus variable number tandem repeats analysis, and by *fim3*, *prn*, and *ptxP* sequence analyses. Strains with 2 closely related SNP profiles carrying *prn2* and *ptxP3* from the recently emerged SNP cluster I predominated. The data suggest increasing selection among the *B. pertussis* population in Australia in favor of strains carrying *prn2* and *ptxP3* under the pressure of acellular vaccine-induced immunity.

Pertussis has reemerged as a significant public health threat in populations with historically high vaccine uptake. The increasing disease rates have been reported in many countries [1–4], including Australia, in recent years. A particularly severe epidemic began in 2008, and at its peak, in 2009, it caused up to 35 000 cases, with an incidence of 156 cases per 100 000 population. In Australia, pertussis vaccine coverage is relatively high, averaging 92% for children aged

<12 months and up to 95% for children aged 24 months [5]. However, in 2008, only 80.7% had received the booster dose scheduled for 5 years of age [5]. During the current epidemic, children aged 5–9 years were most severely affected, even though vaccine coverage has not changed in this age group, at least over the past 8 years [6]. Therefore, other factors in addition to waning vaccine-induced immunity must have contributed to this prolonged epidemic.

Pertussis vaccination in Australia was first implemented using a whole cell vaccine (WCV) during the 1950s, and use of the WCV continued until 1999. Acellular vaccine (ACV) was phased in beginning in 1997, and only ACV has been used since 2000. One ACV contains 3 components: pertussis toxin (Ptx), pertactin (Prn), and filamentous hemagglutinin (Fha); another ACV contains 5 components: Ptx, Prn, Fha, and the fimbrial antigens Fim2 and Fim3. The ACV used predominantly in Australia is the 3-component vaccine [7].

Previously, we characterized 208 Australian *Bordetella pertussis* isolates collected since the 1970s, using 3 molecular typing methods: multilocus variable number of tandem repeats analysis (MLVA) [7], typing of genes encoding antigens used in ACVs (*prn*, *ptxA*, *fim2*, *fim3*, and *fhaB*), and single-nucleotide polymorphism (SNP) typing [8]. We classified SNP types into several SNP clusters and documented an increase in prevalence of clusters I and IV and a decrease in cluster II in Australia after the introduction of ACV. Cluster I contains isolates with MLVA type (MT) 27 and antigenic profile 3 (AP3; *prn2*, *ptxA1*, *fim2-1*, *fim3A*, and *fhaB1*) or AP8 (*prn2*, *ptxA1*, *fim2-1*, *fim3B*, and *fhaB1*); cluster IV consists of isolates with MT70 and AP7 (*prn1*, *ptxA1*, *fim2-2*, *fim3A*, and *fhaB1*). Cluster II was associated with MT29 and AP4 (*prn3*, *ptxA1*, *fim2-1*, *fim3A*, and *fhaB1*). Australian isolates uniformly contained alleles *ptxA1* and *fhaB1*. In this study, we investigated strains of *Bordetella pertussis* recovered during 2008–2010 that are associated with the ongoing Australian epidemic.

## METHODS

We collected 194 clinical *B. pertussis* isolates from 2008 through 2010 from 4 states across mainland Australia, including 77 isolates from New South Wales (NSW), 47 from South Australia (SA), 30 from Victoria (VIC), and 40 from Western Australia (WA). Isolates were obtained from patients living in the capital city of each state, where the majority of the states' residents are located. All available isolates were genotyped. The relatively small number available for

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typing reflects the fact that polymerase chain reaction (PCR) and serology are the preferred diagnostic methods for pertussis, with culture infrequently requested. The total number of cases in 2008–2010 was highest in NSW (29 311 notified cases), followed by SA (14 238 cases), VIC (12 647 cases), and WA (2693 cases). Therefore, the ratio of the number of isolates selected to the total number of cases was similar (2.4–3.3 isolates per 1000) for all states except WA (14.9 isolates per 1000 cases).

Bacterial isolates were inoculated on charcoal agar (Oxoid) supplemented with 10% horse blood and were cultured at 35°C for 3–5 days. DNA was then extracted and used for typing. The isolates were typed by a hairpin real-time PCR-based SNP typing assay [8], by MLVA of 8 variable number tandem repeat (VNTR) loci [7], and by allele typing of antigen genes *prn* and *fim3*, using a real-time PCR-based assay [9]. Isolates were assigned to clusters and SNP profiles (SPs) according to the protocol of Octavia et al [8]. The Simpson diversity index (D) for each typing method was calculated using an in-house program (MLEECOMP; available on request).

## RESULTS

The set of 194 isolates was classified into 11 SPs; the geographic distribution of SPs in different Australian states is shown in Figure 1. Three SPs were from cluster I (13, 14, and 16) and included the majority (86%) of isolates; SP13 (n = 96) was the most common, followed by SP14 (n = 47) and SP16 (n = 24; Figure 2A). Only 2 isolates belonged to cluster II (SP17 and SP37), and the remaining 25 were unclustered (SP1, SP6, SP7, SP9, SP11, and SP18). The frequencies for both SP13 and SP14 were not significantly different across states, and there was no predominance in the distribution of these SPs (Figure 1). Although SP16 was not observed in SA, the overall frequency of SP16 was low, and the absence of SP16 in SA may be due to the small sample size.

MLVA typing resolved the isolates into 27 MTs, of which 9 have been previously observed in our collection of diverse *B. pertussis* isolates and 18 have not (Figure 2B). Of the latter, 7 were novel; that is, they had not been recorded in the publicly available international MLVA database (available at: <http://www.mlva.net>), which contains data for 5 of the 8 VNTR loci (VNTR1, VNTR3, VNTR4, VNTR5, and VNTR6) used in this study. The other 11 were subtypes of the previously reported MTs. The majority of isolates belonged to MT27 (46.3%) or MT214 (13.4%; Figure 2B). The range of different MTs was recorded within the predominant SPs (ie, SP13, SP14, and SP16). For example, 4, 11, 6, and 8 MTs were detected within SP13 isolates collected from patients in NSW, SA, VIC, and WA, respectively. For SP14, NSW and WA had 4 MTs each, while SA and VIC had 5 MTs each. NSW, the largest state in Australia, demonstrated the largest variations in SP16, with 9 MTs; VIC and WA had only 2 MTs each.

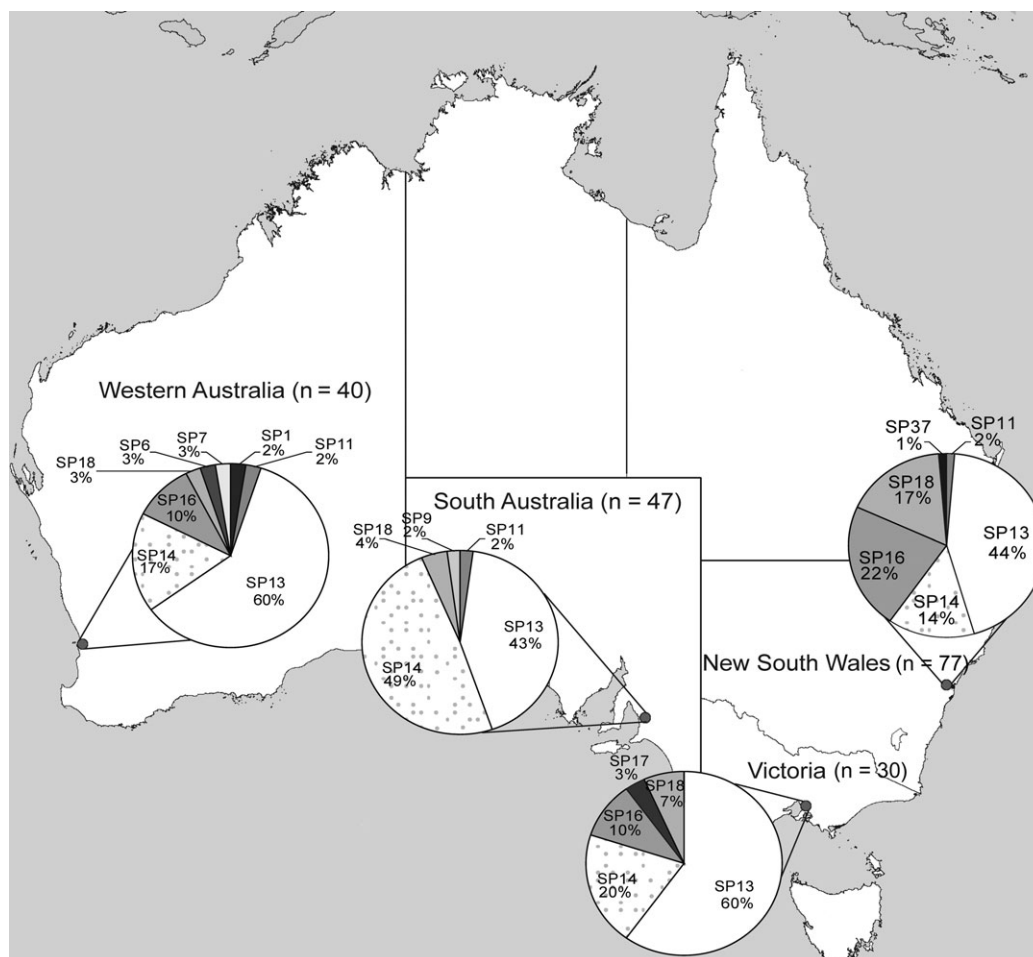
We typed only 2 of the 5 ACV antigen genes *fim3* and *prn* because we previously found that only these 2 antigen genes varied among recent Australian isolates [8]. The majority (141 [73%]) of the 194 isolates had the *fim3A* allele; the remaining 53 had the *fim3B* allele. For *prn*, 167 isolates (86%) carried the *prn2* allele, 20 had *prn1*, 4 had *prn3*, and 1 had *prn7*; *prn* from 2 isolates (L1301 and L1304) was not typeable. We also typed the *ptxP* promoter, which has 18 known alleles, one of which (*ptxP3*) has been associated with increased production of pertussis toxin [10]. A hairpin real-time PCR assay was designed to detect a SNP (G → A) located –65 bp of the *ptxP* region, which is unique to *ptxP3*. Our SNP typing determined the presence or absence of the *ptxP3* allele (non-*ptxP3* alleles include *ptxP1*–*ptxP2* and *ptxP4*–*ptxP19*). We found that 86% of isolates carried *ptxP3*, almost all of which also carried *prn2* (Figure 2C).

The markers analyzed in this study—SNPs, MLVA, *fim3*, *prn*, and *ptxP*—were combined to form a unique polyphasic genotype for each isolate to increase the resolution of subtyping. Overall, the 194 isolates were classified into 46 genotypes. The 77 NSW, 47 SA, 30 VIC, and 40 WA isolates were differentiated into 22, 15, 13, and 19 genotypes, respectively. Overall, the most common genotype (in 22% of isolates) was SP13, MT27, *fim3A*, *prn2*, and *ptxP3*, which represented 29% of isolates from NSW and 30% from VIC. In SA, 34% of isolates were genotype SP14, MT27, *fim3A*, *prn2*, and *ptxP3*. In WA, 33% of isolates were genotype SP13, MT214, *fim3A*, *prn2*, and *ptxP3*. VIC isolates were the most diverse (D = 0.887), followed by NSW (D = 0.878), WA (D = 0.869), and SA (D = 0.848). The distribution of genotypes was similar among the isolates from the eastern states of NSW and VIC and differed most from SA isolates.

## DISCUSSION

*B. pertussis* isolates collected from 4 Australian states during an ongoing pertussis epidemic that began in 2008 were classified using SNPs, MLVA, *fim3*, *prn*, and *ptxP* typing. SNP cluster I strains, primarily SP13 and SP14, accounted for 86% of isolates (Figure 2A). This is a significant increase from our previous study of isolates collected between 2000 and 2007, in which SNP cluster I represented only 31% of isolates. This suggests increasing selection among the *B. pertussis* population in Australia in favor of strains carrying antigens that differ from those represented in ACVs.

There was no significant difference in the distribution of SPs across the 4 Australian states from which isolates were collected. We have previously shown that isolates with SP13 and SP14 were isolated as early as 2000 [8]. This suggests that they were circulating for at least 8 years before causing the epidemic that currently exists throughout Australia.



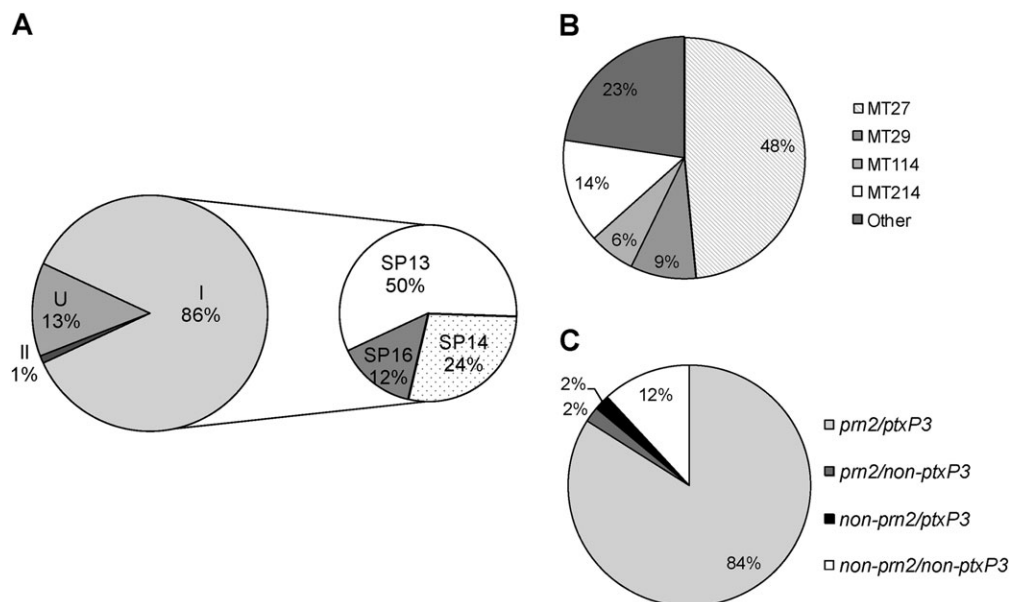
**Figure 1.** Distribution of epidemic isolates recovered during 2008–2010, by single nucleotide polymorphism (SNP) profiles (SPs), across the capital cities of 4 states in Australia.

The combination of *prn* and *ptxP* alleles is of particular importance. The isolates were divided into 4 types: *prn2-ptx3*, non-*prn2*-non-*ptxP3*, *prn2*-non-*ptxP3*, and non-*prn2*-*ptxP3*. The latter 2 had a low overall frequency of 4%. However, 84% of the isolates were *prn2-ptxP3* (Figure 2C). Previous immunological evidence clearly has shown that *prn2* has an advantage against ACV immune selection pressure [11, 12], while *ptxP3* has been found to be associated with higher virulence on the basis of hospitalization and case mortality data [10]. Although isolates from this study were not typed for *ptxA*, it should be noted that, on the basis of data from our previous study, all cluster I–IV strains carry *ptxA1*. This means that there is an allelic mismatch between the currently circulating (SNP cluster I) *ptxA* allele, *ptxA1*, and the ACV allele, *ptxA2*. Increased production of PT promoted by *ptxP3* may compound the effect of the antigenic difference between the products of different *ptxA* alleles in reducing ACV-induced protection. Clonal expansion of *B. pertussis* strains carrying *ptxP3* has also been associated with recent epidemics of pertussis

in several European countries [1, 13]. Strains carrying *prn2*, some of which presumably also carry *ptxP3*, have been found or have increased in frequency in China [14] and other countries [13] where WCVs are used. It remains to be seen whether these strains will displace the others in WCV-immunized populations.

The *prn2-ptxP3* isolates have the potential not only to evade the protective effects of ACV but also to increase disease severity as a double act of *B. pertussis* adaptation. Therefore, vaccine-induced selection could contribute to the expansion of cluster I, specifically SP13 and SP14, because of the presence of both *prn2* and *ptxP3*. These 2 SPs have swept across Australia during the epidemic period. Interestingly, they have also been found in other countries [8], suggesting that they have the potential to cause epidemics elsewhere. Therefore, it is very important to monitor the prevalence of these clones globally.

Our previous study showed that cluster II decreased in frequency after introduction of ACV [8]. Current data suggest



**Figure 2.** Distribution of single nucleotide polymorphism (SNP) clusters and profiles, multilocus variable number of tandem repeats analysis types (MTs) and *prn-ptxP* alleles among epidemic isolates recovered during 2008–2010 in Australia. **A**, Distribution of isolates by SNP clusters (I, II, and U [unclustered]) and SNP profiles (SPs) within cluster I. **B**, Distribution of isolates by MTs, with the top 4 MTs shown. **C**, Distribution of *prn* and *ptxP* types.

that this cluster is on the verge of being completely replaced by cluster I. Cluster IV also increased after introduction of ACV in our previous study [8]. However, it was not observed in this more recent epidemic in the 4 states we sampled. More samples from the other states may help to explain the deficiency of cluster IV strains in this study. Furthermore, this study lays the foundation for future studies to determine any correlation between genotypes of *B. pertussis* isolates and both disease severity and vaccination history.

In conclusion, the prolonged epidemic in Australia that began in 2008 was predominantly caused by SPs from cluster I carrying *prn2* and *ptxP3*, which have been circulating in this country since at least 2000. There may be other unknown factors contributing to the increase of cluster I strains, but they appear to have a selective advantage over strains carrying non-*prn2* and non-*ptxP3* alleles under the pressure of ACV vaccination. Therefore, clones carrying *prn2-ptxP3* have the potential to cause epidemics in other countries covered by ACV with formulations similar to those used in Australia and should be monitored locally and globally.

## Notes

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