Epidemiol. Infect. (2004), **132**, 185–193. © 2003 Cambridge University Press
DOI: 10.1017/S095026880300164X Printed in the United Kingdom

Temporal trends in circulating *Bordetella pertussis* strains in Australia

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(*Accepted 7 October 2003*)

SUMMARY

Australia experienced a resurgence of pertussis in the 1990s despite improved vaccine coverage. Although much of the increase was attributable to increased detection of cases in older persons with waning immunity by serology, vaccine changes or alterations in circulating *Bordetella pertussis* strains may also have contributed. We determined the frequency of variants of *B. pertussis* pertactin (*prn*), and pertussis toxin subunit 1 (*ptxS1*) genes, restriction fragment length polymorphism (RFLP) types and fimbrial serotypes prevalent in Australia prior to, and during the 1990s. Ampoules of the whole-cell vaccine in use prior to 1999 and 84 *B. pertussis* isolates stored between 1967 and 1998 by laboratories around Australia were analysed. One pertactin allele, *Prn3*, not detected before 1985, was found in 24 out of 57 (42%) isolates between 1989 and 1998 ($P<0{\cdot}0001$). *PtxS1A* was found in all isolates. IS*1002* type 29, found in 17 out of 31 (55%) isolates tested, was the predominant RFLP type. The only difference in fimbrial serotype distribution between the time-periods was an increase in serotype 3 ($P=0{\cdot}054$). The whole-cell vaccine contained only the alleles *prn1* and *ptxS1A*. Antigenic shift in *B. pertussis* may have contributed to the re-emergence of pertussis in Australia. Monitoring these trends will be important as acellular vaccines are introduced and changes are made to pertussis vaccine schedules.

INTRODUCTION

Australia, together with other industrialized countries such as The Netherlands, Canada and the United States, has experienced a resurgence of pertussis in the past decade, despite improved vaccine coverage (Fig. 1) [1]. In Australia, the crude pertussis notification rate rose from 2·0 to 58·9 per 10^5 population between 1991 and 1997 [2]. Notifications of pertussis peaked in 1997, with 10 907 cases identified in that year [1]. There has also been a shift in age-specific incidence of pertussis, with an increase in the median age of notification from 4 years in 1984 to 15 years in 1996 and 21 years in 1998 [3]. Until 1999, infants had the highest pertussis notification rate. Since then the highest notification rate has been in the 10–14 years age group [4].

In highly immunized populations, apparent increases in the incidence of pertussis may be related to increased detection, an increase in the incidence or

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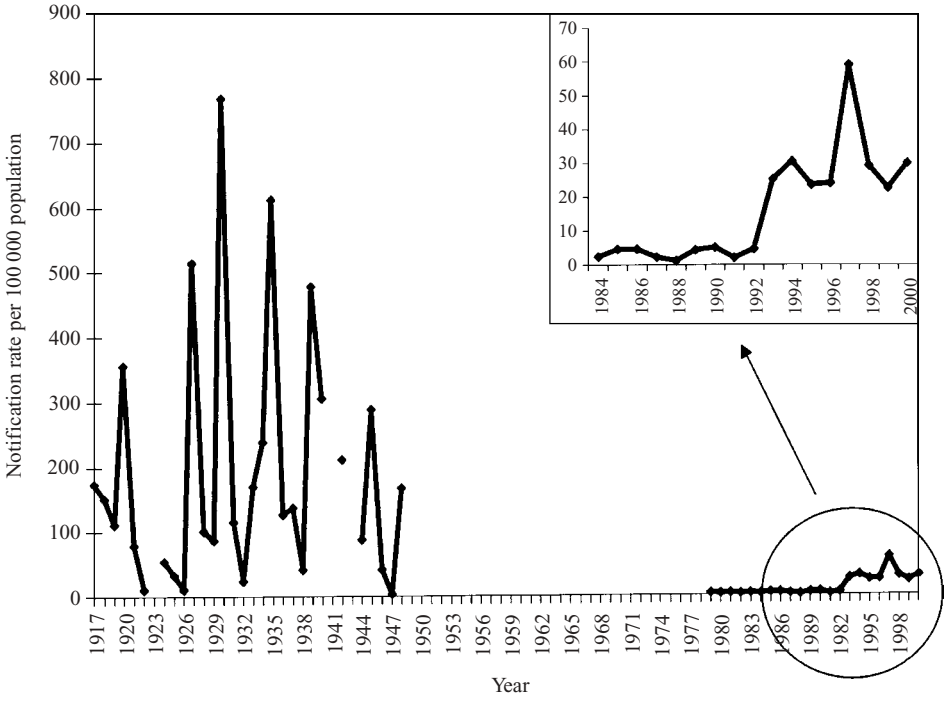

Fig. 1. Annual adjusted pertussis notification rate in Australia, 1917–2000.

severity of *B. pertussis* infection, or both. Increased detection may be through improved surveillance or changes in diagnostic practice, while an increased incidence could be related to the vaccine in use (changes in vaccine quality or population coverage) or alterations in circulating *B. pertussis* strains [5, 6]. In Australia, a number of these factors may have contributed to the increase in pertussis notifications. First, surveillance of communicable diseases at national level was enhanced by the introduction in 1991 of the National Notifiable Diseases Surveillance System [2]. Second, and possibly most importantly, a locally produced enzyme immunoassay for IgA against whole-cell sonicated *B. pertussis* antigen (BPIgA) became widely available in the early 1990s. This coincided with most Australian jurisdictions accepting laboratory notification of pertussis based on a single high antibody titre. As BPIgA appears to be specific for clinical pertussis, the incidence of pertussis is unlikely to have been over-estimated by acceptance of laboratory notifications [7].

Third, there is evidence of a real increase in pertussis disease in infants, which is seldom diagnosed serologically. Throughout Australia, between 1995 and 2000, 10 infants died from pertussis including 6 in 1997; this is more than the total number of pertussis deaths recorded in Australia over the previous 20 years [4]. Fourth, a significant increase in hospitalizations coded as pertussis in people over the age of 15 years during the 1990s, and not seen in younger age groups, is less likely to be attributable to increased serological diagnosis than notifications [8]. Together, these developments suggest that a real increase in pertussis activity has occurred, despite an increase in vaccine coverage over the previous decade [9].

Changes in circulating *B. pertussis* strains have been implicated in pertussis epidemics in The Netherlands, with an increase in the proportion of cases in vaccinated children from 55% in 1989 to 85% in 1996, in the presence of high whole-cell vaccine coverage [10–12]. In Australia, a decrease over time in the effectiveness of the local whole-cell pertussis vaccine, in use since the 1950s, may also have contributed to increased pertussis incidence, related to a mismatch between vaccine and circulating strains. In this study, we investigated whether similar changes in circulating *B. pertussis* strains to those seen in The Netherlands were temporally associated with the re-emergence of pertussis in Australia.

METHODS

Study population

No systematic collection of *B. pertussis* isolates was available for examination. A total of 84 *B. pertussis*

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isolates were identified by laboratories in urban centres in the five largest Australian states: Perth (Western Australia), 14; Brisbane (Queensland), 22; Melbourne (Victoria), 11; Adelaide (South Australia), 10; Sydney and Newcastle (New South Wales), 27. The years of isolation ranged from 1967 to 1998.

Isolates were forwarded to the National Centre for Immunisation Research and Surveillance of Vaccine-Preventable Diseases (NCIRS), Sydney, Australia for transport as a batch to the National Institute for Public Health and the Environment (RIVM), Bilthoven, The Netherlands. *B. pertussis* strains used for the manufacture of the whole-cell pertussis vaccine in use in Australia were not available for analysis, so ampoules of the whole-cell diphtheria, tetanus, pertussis (DTP) vaccine in use in Australia, manufactured by the Commonwealth Serum Laboratories (CSL Ltd, Melbourne), were sent to RIVM.

Microbiological methods

B. pertussis strains were grown on Bordet–Gengou (BG) agar (Difco Catalogue no. 0048-17-5, Detroit, MI, USA) supplemented with 1% glycerol and 15% sheep blood at 35 °C for 3 days.

DNA extraction, PCR amplification and sequencing

The method of DNA extraction from fresh cultures of *B. pertussis*, primers, PCR protocols and sequencing methods have been described previously [5]. PCR was performed directly on freeze-dried isolates that could not be subcultured and on the DTP vaccine formulation. PCR fragments were purified with a Qiaquick (Qiagen) PCR purification kit and sequenced on both strands using the amplification primers in combination with internal primers. Sequence reactions were carried out with an ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction kit and the products were analysed on a model 373 or 377 ABI DNA sequencer (PerkinElmer Applied Biosystems).

Pertactin (*prn*) gene PCR

Approximately 400 base-pair (bp) fragments of two previously identified repetitive regions of the *prn* gene, designated 1 and 2 [5], were chosen for sequencing. The repeat regions 1 and 2 comprise 70 and 40 bases respectively. As variation is usually restricted to region 1, this region was sequenced in all strains whereas region 2 was sequenced in 1 of 7 strains. The novel *prn* allele identified was sequenced completely.

PtxS1 subunit of pertussis toxin gene PCR

The *ptxS1* subunit of the pertussis toxin gene was sequenced in its entirety. Two novel alleles, *ptxS1F*, *ptxS1G*, were submitted to the EMBL bank.

IS*1002* DNA fingerprinting

IS*1002*-based DNA fingerprinting was performed as described previously by digesting chromosomal DNA with *Sma*I [11]. Samples were analysed on agarose gels (Pulse Field Certified; Bio-Rad, Hercules, CA, USA), and transferred to Hybond N-membrane (Amersham Pharmacia Biotech, Bucks., UK) using standard DNA blotting techniques. A 293 bp IS*1002* probe was used for hybridization. Labelling of the probe with peroxidase and detection of hybridizing bands was performed according to the instructions of the Enhanced Chemiluminescence Gene Detection System (Amersham Pharmacia Biotech). Exposed films were scanned at 190 dpi (HP Scanjet IIcx/T; Hewlett-Packard, Foster City, CA, USA). Films were analysed using Bionumerics software (Applied Maths, Sint-Martens-Latem). Assignment of IS*1002* fingerprint patterns to similarity groups was performed by the calculation of pair-wise similarities using the Dice coefficient and cluster analysis with the UPGMA algorithm.

Serotyping

On a grease-free slide, bacterial colonies were emulsified in a drop of in-house Fim2 or Fim3 polyclonal rabbit antiserum, after which agglutination was determined macroscopically. To test for autoagglutination the drop of serum was replaced by physiological saline.

Data analysis

Differences in the proportions of *prn* types, *ptxS1* types, IS*1002* restriction fragment length polymorphism (RFLP) types and serotypes between two time-periods (before and after 1989) were calculated using Fisher's exact test (two-sided). These two time-periods were chosen because of the observed increase in pertussis incidence in the 1990s and the time-periods for available isolates (1967–1985 and 1989–1998).

Table 1. *Proportion of* B. pertussis *isolates typed in each time-period*

Time-period	Isolates typed (any method)	Isolates typed for *prn*	Isolates typed for *ptxS1*	Isolates serotyped	Isolates RFLP typed (IS*1002*)
1967–1985	26	26	19	15	16
1989–1998	58	57	43	12	15
Total	84	83	62	27	31

RESULTS

Geographic and demographic patterns of *B. pertussis* isolates

Eighty-four *B. pertussis* strains were examined for one or more of pertactin type, pertussis toxin S1 type, serotype and IS*1002* type (Table 1). Strains were a non-random, representative selection from each of the years and cities. The geographic distribution of the *B. pertussis* strains submitted is shown in Figure 2.

Demographic data were available for only a minority of patients. Of these, 15 out of 34 (44·1%) were aged less than 6 months, 12 out of 22 (54·5%) were female and 6 out of 9 (66·7%) had received no immunizations. It is likely that most isolates came from infants and children, as a number of the referring laboratories (Perth, Adelaide, Melbourne and Sydney) service paediatric hospitals. All *B. pertussis* isolates originating from Melbourne, Brisbane and Newcastle were from 1989 onwards.

Pertactin types

The Australian whole-cell vaccine formulation contained the *prn1* allele, in common with most whole-cell vaccines analysed [5]. Four *prn* types (*prn1*, *prn2*, *prn3* and *prn11*) were identified among *B. pertussis* strains isolated between 1967 and 1998. Prior to 1989, *prn1* was the most common (21/26 isolates, 81%) with no *prn2* or *prn3* identified.

A novel type, *prn11*, which had not been detected elsewhere previously, was found in 5 out of 26 isolates (19%) prior to 1989. Both regions (1 and 2), and the complete gene from one isolate, were fully sequenced and the sequence submitted to EMBL (accession no. AJ507642). Variation was limited to region 1, as observed with most *prn* variants [13]. Two of the five isolates with the *prn11* allele had identical IS*1002*, *ptxS1* and serotype. Since all were from Perth and isolated in the same year (1982), it is likely that they were epidemiologically related.

Among isolates from 1989 onwards, 24 out of 57 (42%) contained *prn3*, not detected prior to 1985. Of these 24, 10 (42%) originated from one centre (Brisbane). The increase in the proportion of isolates containing *prn3* and decrease in the proportion of isolates containing *prn1* between the periods 1967–1985 and 1989–1998, was highly significant (Table 2). As inclusion of isolates from geographic areas not represented in both time-periods may have introduced bias, an analysis restricted to the 24 isolates from Perth, Adelaide and Sydney (centres represented in both time-periods) was performed. In this sub-group, the proportion of isolates containing *prn3* in 1989–1998 remained significantly higher than in 1967–1985 (10/24 *vs.* 0/26, $P = 0{\cdot}003$, Fisher's exact test).

Pertussis toxin S1 type

The Australian whole-cell vaccine formulation contained the *ptxS1A* allele, as did almost all (92%) of the 62 *B. pertussis* isolates examined for pertussis toxin S1 type. Two novel *ptxS1* alleles were identified, *ptxS1F* and *ptxS1G* and submitted to the EMBL bank (accession nos. AJ506994 and AJ506995 respectively). As both these alleles contained silent mutations relative to *ptxS1A* [5], the predicted protein encoded by all three alleles would be identical, so all strains analysed produced a protein identical to PtxS1A.

Serotypes

In the first period, from 1967 to 1985, serotype 2 predominated, being present in 9 out of 15 (60%) strains analysed, with serotypes 3 and 2,3 found in 5 (33%) and 1 (7%) of the strains respectively. After 1989, there was an increase in the proportion of serotype 3 isolates, from 5 out of 15 (33%) to 9 out of 12 (75%) strains (Table 2).

https://doi.org/10.1017/S095026880300164X Published online by Cambridge University Press

Table 2. *Characteristics of* B. pertussis *strains from time-periods 1967–1985 and 1989–1998 by* prn *type,* ptxS1 *type and serotype*

Time-period	Isolates typed*	*prn1*‡ *n* (%)†	*prn2* *n* (%)†	*prn3*§ *n* (%)†	*prn11* *n* (%)†	*ptxS1A* *n* (%)†	Serotype 2 *n* (%)†	Serotype 2,3 *n* (%)†	Serotype 3‖ *n* (%)†
1967–1985									
n/total	26	21/26	0/26	0/26	5/26	19/19	9/15	1/15	5/15
(%)		(81%)	(0%)	(0%)	(19%)	(100%)	(60%)	(7%)	(33%)
1989–1998									
n/total	58	27/57	6/57	24/57	0/57	43/43	3/12	0/12	9/12
(%)		(47%)	(11%)	(42%)	(0%)	(100%)	(25%)	(0%)	(75%)
Total	84	48	6	24	5	62	12	1	14

* Total isolates typed by any method.
† Percentage of each specific type (*prn*, *ptxS1A*, serotype) in each time-period.
‡ $P = 0{\cdot}005$, comparing proportions of isolates containing *prn1* in 1967–1985 with 1989–1998.
§ $P < 0{\cdot}0001$, comparing proportions of isolates containing *prn3* in 1967–1985 with 1989–1998.
‖ $P = 0{\cdot}054$, comparing the proportion of serotype 3 isolates in 1967–1985 with 1989–1998.

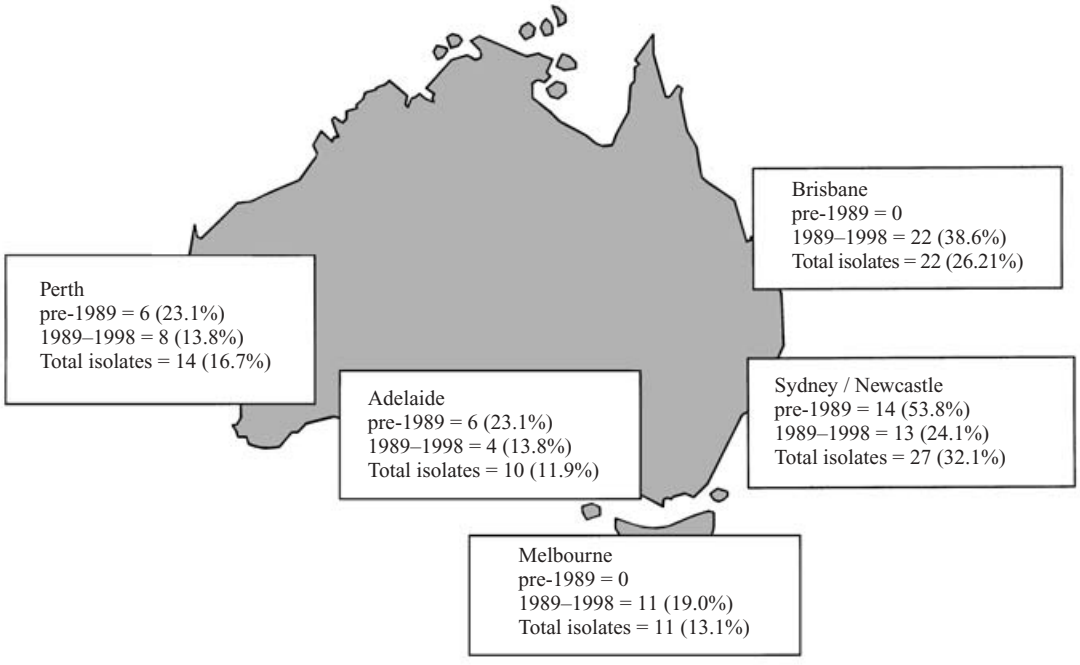

Fig. 2. Origin of Australian *B. pertussis* isolates from 1967 to 1999 ($n = 84$) (pre-1989, 26; 1989–1998, 58).

IS*1002* types

Of the 31 isolates that were DNA-typed using IS*1002*-based RFLP, 17 (54·8%) were IS*1002* type 29, which was predominant in both time-periods, 1967–1985 and 1989–1998. IS*1002* type 55 was the next most frequent type (five isolates, 16%) (Table 3).

DISCUSSION

In Australia, CSL Ltd first manufactured whole-cell pertussis vaccines in 1920. Although no trials were conducted for whole-cell vaccine efficacy in children in Australia [2, 14] mass vaccination using a DTP combination commenced in the early 1950s and was followed by a dramatic reduction in the numbers of notified cases [1–4]. Pertussis was not notifiable in all jurisdictions in the Australian federation until 1991, but data from South Australia showed low rates of notification over the intervening period (Fig. 1) [3].

In 1978–1979, there was a change in the vaccine schedule, with the removal of the fourth dose at 18 months due to concern about adverse effects. This dose was subsequently reintroduced in 1985, after

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Table 3. *Characteristics of* B. pertussis *strains from time-periods 1967–1985 and 1989–1998 by IS*1002 *type*

Time-period	Isolates typed by RFLP	IS*1002* type 12	IS*1002* type 19	IS*1002* type 29*	IS*1002* type 33	IS*1002* type 35	IS*1002* type 55	IS*1002* type 83
1967–1985								
n/total (%)	16	2/16 (13%)	2/16 (13%)	10/16 (63%)	2/16 (13%)	0/16 (0%)	0/16 (0%)	0/16 (0%)
1989–1998								
n/total (%)	15	0/15 (0%)	1/15 (7%)	7/15 (47%)	0/15 (0%)	1/15 (6%)	5/15 (33%)	1/15 (6%)
Total	31	2	3	17	2	1	5	1

* $P = 0{\cdot}479$, comparing proportions of isolates containing IS*1001* type 29 in 1967–1985 with 1989–1998.

concern about increased cases in young children. A fifth dose for children aged 4–5 years was introduced in 1994 [1, 14]. The isolates in this study predate acellular pertussis vaccines, which first became available in Australia in 1997. Acellular pertussis vaccines replaced whole-cell vaccines in the national immunization schedule for the fourth and fifth booster doses in 1998 and for primary pertussis vaccination in 1999.

There were major increases in DTP vaccine coverage in Australia during the decade 1985–1995 [9]. In 1983, national coverage for three or more doses of DTP in children aged between 2 and 5 years was estimated to be only 34% [15]. By 1989, this had increased to 70% of children aged between 0 and 6 years and by 1995, to 87% of children aged between 7 and 24 months [9, 16]. The only recent estimate of the effectiveness of the Australian whole-cell vaccine in infants is 91% (95% CI 86–94), falling to 78% among the 9–13 years age group, comparable to similar screening method estimates of whole-cell pertussis vaccine effectiveness in other countries [17]. These data suggest that the increase in pertussis notifications seen in the 1990s is unlikely to be explained completely either by decreased pertussis vaccine coverage or by major inefficacy of the locally manufactured whole-cell vaccine. However, it is possible that vaccine effectiveness could have diminished compared to historical levels, as no earlier estimates are available.

Direct sequencing of PCR products revealed that the Australian whole-cell vaccine contained *prn1* and *ptxS1A*. Some other whole-cell vaccines analysed also contained *Prn1* [5, 18–21]. However, other whole-cell vaccines, with the exception of the UK vaccine, differed from the Australian vaccine in that they contained *ptxS1B* or *ptxS1D* [18–20], pertussis toxin types not detected in any of the Australian isolates. Among the strains examined in this study, *prn3*, a *prn* variant not present in the whole-cell vaccine used for primary childhood immunization, appeared between 1985 and 1989. It was found in approximately 40% of strains after 1989 and was not confined to any one geographic area. In Europe, *prn2* preceded *prn3* and is the predominant *prn* type in most countries. However, *prn2* was not identified in this sample of Australian isolates until 1996.

An analysis of *B. pertussis* strains from The Netherlands from 1949 to 1996 found that the marked increase in the incidence of pertussis was preceded by the emergence of strains with altered *prn* and *ptxS1* [5]. In contrast to The Netherlands, neither a comprehensive panel of isolates nor complete longitudinal notification data for pertussis were available in Australia during the whole study period. By 1990, non-vaccine *prn* types and *ptxS1* types were found in 80% of strains in The Netherlands, suggesting selection by vaccination, possibly resulting in reduced vaccine efficacy [5]. In The Netherlands, *prn2* and *prn3* were first detected in 1981, and reached predominance in 1992. However, notifications did not rise dramatically until 1996.

Prn3 was first detected in 1989 in Australia, and as in The Netherlands, notifications of pertussis did not rise dramatically until a few years later (1993). Although a decrease in vaccine efficacy would not result immediately in a pertussis epidemic, as it takes time for the susceptible population to reach a critical level, increased notifications were most prominent in older children and adults [1]. This suggests that waning vaccine-induced immunity and poorer historical pertussis vaccine coverage are also factors in the resurgence of pertussis in Australia. Now that acellular vaccines have replaced whole-cell vaccines in the Australian immunization schedule, the identification of circulating *prn* variants may be more

important, as acellular vaccines contain only a restricted range of antigens.

A novel *prn* type (*prn11*) was found in five strains from one geographically isolated region (Western Australia) in one year (1982). As observed with most other *prn* types, variation was restricted to region 1. *Prn11* was not detected in any other region or time-period. Among other pertussis antigenic variants, *ptxS1A* (100% of isolates tested) was predominant. *PtxS1A* also dominates in Europe and was present in 88% of Dutch isolates from 1990 to 1996 [5] and 98% of clinical isolates from the United States from 1996 to 1999 [22]. IS*1002* type 29 (55% of isolates tested) was the predominant chromosomal variant, while IS*1002* types 29 and 35 are also prevalent in The Netherlands [11].

Although only small numbers of isolates were serotyped, the only notable difference in serotype distribution between 1967–1985 and 1989–1998 was an increase in serotype 3 after 1989. An earlier study of 252 Australian field strains and 33 strains used for the production of vaccines from 1950 to 1970 also found changes over time in serotype composition. In early 1964 (when vaccines were low in serotype 1,2 antigens) an upsurge of field isolates of these serotypes was observed. Following a change in vaccine composition in mid-1964 to include serotype 1,2 strains, the number of serotype 1,2 field isolates fell [23]. However, strains isolated between 1981 and 1990 also demonstrated predominance of serotype 1,2 until 1987, followed by serotype 1,3 predominance from 1987 to 1990 [14].

The apparent relationship between characteristics of *B. pertussis* populations and the effectiveness of the different vaccine schedules and vaccines has varied between countries [6]. Large numbers of pertussis notifications in Poland in 1997 and 1998 led to the analysis of clinical isolates. *Prn2* and *prn4* appeared after 1995 and vaccine-type pertussis toxin (*ptxS1B*) was replaced by *ptxS1A* [20]. Finland has a pertussis vaccination history similar to The Netherlands, with a long period of very high population coverage and a schedule comprising four doses. Despite the predominance of non-vaccine type *prn*, there was no evidence of an increase in incidence of pertussis to the extent found in The Netherlands [18]. Similar findings have been observed in France [24].

In the United Kingdom, which has a high vaccination coverage and a low incidence of pertussis, isolates from 1920 to 1999 were analysed. The emergence of non-vaccine variants of *prn* (*prn2* and *prn3*) from the 1980s onwards was demonstrated. Compared to Finland and The Netherlands, the frequency of the vaccine-type *prn* (*prn1*) was much higher ($\sim$50%) in the 1990s [21]. In Italy, a country with low vaccine coverage, four *prn* variants (*prn1*, *prn2*, *prn3* and *prn5*) were found in clinical strains and *prn1* was present in vaccine strains. The frequency of the *prn1* variant was lower in vaccinated than unvaccinated cases of pertussis [19].

In the United States, analysis of clinical *B. pertussis* isolates demonstrated a shift in *prn* variants (the emergence and subsequent predominance of *prn2* and *ptxS1A* from the 1970s) in the setting of an increased incidence of pertussis amongst adolescents aged 10–19 years. However, because of the large number and diversity of vaccines available, evaluation of the components of whole-cell vaccine and comparison with clinical isolates was not possible [22]. Thus, the emergence and predominance of new or non-vaccine *prn* and *ptxS1* variants in clinical isolates has not been uniformly accompanied by an increased incidence of pertussis.

The limitations of this study include the lack of patient data (age, sex and immunization status) for isolates and the opportunistic nature of the sample of strains available for typing. However, the change in pertactin-type distribution was striking and remained significant when restricted to regions with strains available from both time-periods. Importantly, the presence of pertactin antibodies has been shown to have a significant role in protection against infection with *B. pertussis* [25, 26].

In conclusion, there is evidence of an antigenic shift in *B. pertussis* strains over the past 30 years in Australia, similar to other countries with long-standing whole-cell vaccine use. Although there is evidence of a real increase in pertussis, as in other countries, there is no direct relationship between this and the timing of strain changes. While difficult to disentangle from other factors, a progressively increasing mismatch between circulating strains and those in the whole-cell vaccine then in use may have contributed to this increase. It will be important to maintain molecular surveillance of prevalent pertussis strains, along with standard disease surveillance, in the context of the recent major changes to the available vaccines and schedules of vaccine administration in Australia.

ACKNOWLEDGEMENTS

The authors thank Dr John Ferguson, John Hunter Hospital, Newcastle, for providing clinical *B. pertussis*

isolates and for his constructive comments. They also thank Dr Joan Faoagali, Royal Brisbane Hospital, Brisbane; Associate Professor Geoff Hogg, University of Melbourne, Melbourne; Dr Tony Keil, Princess Margaret Hospital for Children, Perth; Professor John Turnidge, Women's and Children's Hospital, Adelaide and Associate Professor William Rawlinson, Prince of Wales Hospital, Sydney, for providing clinical *B. pertussis* isolates. Without this national collaboration, the study would not have been possible. The National Centre for Immunisation Research and Surveillance of Vaccine-Preventable Diseases is supported by The Australian Government Department of Health and Ageing, The New South Wales Department of Health and The Children's Hospital at Westmead.

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