Antigenic variants in *Bordetella pertussis* strains isolated from vaccinated and unvaccinated children

Paola Mastrantonio,¹ Patrizia Spigaglia,¹ Hans van Oirschot,²
Han G. J. van der Heide,² Kees Heuvelman,² Paola Stefanelli¹
and Frits R. Mooi²

Author for correspondence: Paola Mastrantonio. Tel: +39 06 49902335. Fax: +39 06 49387112.
e-mail: pmastran@iss.it

¹ Department of Bacteriology and Medical Mycology, Istituto Superiore di Sanità, Viale Regina Elena 299, 00161 Rome, Italy

² Research Laboratory for Infectious Diseases, National Institute of Public Health and the Environment, Bilthoven, The Netherlands

***Bordetella pertussis* shows polymorphism in two proteins, pertactin (Prn) and the pertussis toxin (PT) S1 subunit, which are important for immunity. A previous study has shown antigenic shifts in these proteins in the Dutch *B. pertussis* population, and it was suggested that these shifts were driven by vaccination. The recent Italian clinical trial provided the opportunity to compare the frequencies of Prn and PT S1 subunit variants in strains isolated from unvaccinated children, and from children vaccinated with two acellular and one whole-cell pertussis vaccine. Four Prn variants (Prn1, Prn2, Prn3 and Prn5) were found in the 129 strains analysed. Prn1, Prn2 and Prn3 have been described previously, whereas Prn5 is a novel variant. Prn1, Prn2, Prn3 and Prn5 were found in, respectively, 6, 41, 51 and 2% of the strains. The *B. pertussis* strains used to produce the vaccines administered in the clinical trial were found to produce Prn1, or a type which differed from Prn1 in one amino acid. The frequency of the Prn1 variant was found to be lowest in the strains isolated from vaccinated groups, suggesting that Prn1 strains are more affected by vaccine-induced immunity than Prn2 and Prn3 strains. Only one PT S1 type (S1A) was observed in the examined strains, which was distinct from the types produced by the vaccine strains (S1B and S1D). The S1A type also predominates in the Dutch *B. pertussis* population. The genetic relationship among *B. pertussis* strains analysed by IS1002-based DNA fingerprinting revealed that three fingerprint types predominate, representing more than 70% of the strains. Prn2 strains showed a greater variety of fingerprint types compared to Prn3, suggesting that Prn3 has emerged more recently. The results are discussed in the light of vaccine-driven evolution.**

Keywords: *Bordetella pertussis*, protective antigens, polymorphism

INTRODUCTION

Bordetella pertussis, the aetiological agent of whooping cough, produces many virulence factors (Leininger *et al.*, 1990, 1991; Relman *et al.*, 1989; Weiss & Hewlett, 1986). A number of these factors, pertussis toxin (PT), filamentous haemagglutinin, pertactin (Prn) and fimbriae, are proteins involved in intoxication and/or

adhesion processes to host tissues and are critical antigens responsible for inducing immunity to *B. pertussis*.

Results obtained in studies with experimental animal models showed that these antigens are efficacious in preventing *B. pertussis* infections, thus supporting their use in the new generation of acellular pertussis vaccines (Cahill *et al.*, 1993; Edwards, 1993; Kimura & Kuno-Sakai, 1990; Peppoloni *et al.*, 1995; Shahin *et al.*, 1990).

Recent clinical trials, successfully conducted in countries with a traditionally low rate of vaccination with the

Abbreviations: Prn, pertactin; PT, pertussis toxin.

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whole-cell pertussis vaccine, demonstrated the high level of efficacy of some new acellular pertussis vaccines containing the above-mentioned antigens (Ad Hoc Group for the Study of Pertussis Vaccines, 1988; Greco *et al.*, 1996; Trollfors *et al.*, 1995; Willems & Mooi, 1996).

The resurgence of pertussis experienced during recent years in countries with a long tradition in pertussis vaccination, such as the United States, the Netherlands, Canada and Australia (Andrews *et al.*, 1997; Bass & Wittler, 1994; de Melker *et al.*, 1997; De Serres *et al.*, 1995), prompted researchers to determine whether vaccination had affected the population structure of *B. pertussis* and had resulted in antigenic shifts (Mooi *et al.*, 1998; van der Zee *et al.*, 1996). The results of these recent studies indicate a shift in the population structure of strains isolated in successive time periods and a divergence in the PT and Prn proteins between vaccine strains and clinical isolates. In particular, three Prn variants (Prn1, Prn2 and Prn3) and four PT S1 subunit variants (S1A, S1B, S1C and S1D) were identified. The variants showed differences at the amino acid level, and the epidemiological data suggest that these changes may have been driven by vaccination (Mooi *et al.*, 1998).

The availability of strains collected during the recent Italian field trial (Greco *et al.*, 1996; Mastrantonio *et al.*, 1995), in which the efficacy of a number of pertussis vaccines was evaluated, provided a unique opportunity to compare strains isolated from vaccinated and unvaccinated children. Furthermore, it allowed us to compare strains from regions which, in the years before the beginning of the trial, presented different vaccine coverages (Cinqueti *et al.*, 1994; Lo Palco *et al.*, 1995).

METHODS

Study population. Starting in 1993, 15 601 infants at the age of 2 months were enrolled in a double-blind clinical trial to evaluate the efficacy of two new acellular pertussis vaccines.

Three pertussis vaccines combined with diphtheria and tetanus toxoids (DTP) were included: the whole-cell DTP vaccine manufactured by Connaught Lab and two acellular vaccines (DTPa), one manufactured by Chiron and the other by SmithKline Beecham Biologicals (SKB). Both the acellular vaccines contained three pertussis antigens: PT, filamentous haemagglutinin and Prn.

A fourth vaccine arm in the trial, from now on referred to as the unvaccinated group, was made up of 1555 children who received only diphtheria and tetanus toxoids. The trial study design and its results, previously published (Greco *et al.*, 1996), showed that each of the two acellular vaccines was 84% efficacious after three doses whereas the efficacy of the whole-cell DTP vaccine was 36%.

The active surveillance of the cohorts of children which received acellular vaccines is still ongoing through a mean age of 5 years, maintaining the same case definition and methods of case ascertainment (Salmaso *et al.*, 1998).

Bacterial strains. One hundred and twenty nine *B. pertussis* strains out of 344 isolated in the years 1993–1995 from cases

occurring in the unvaccinated and vaccinated groups have been investigated for the present study. Thirty strains were isolated from recipients of DTPa SKB, 27 from recipients of the DTPa Chiron, 24 from the Connaught whole-cell DTP vaccine recipients and 48 from unvaccinated children. These strains were isolated in the four different regions participating in the trial: Friuli ($n=4$), Veneto ($n=37$), Piemonte ($n=37$) and Puglia ($n=51$). All the strains were isolated from cases fulfilling the WHO clinical case definition of at least 21 d of paroxysmal cough.

The analysis was initially performed on an equal percentage of randomly chosen strains, isolated from vaccinated and unvaccinated children and collected in equal proportions in the Northern regions which had higher pre-trial vaccination coverage (Piemonte, Veneto and Friuli) and in the Southern region of Puglia with a lower pre-trial vaccination coverage. A further 24 *B. pertussis* strains isolated in Puglia were later added to the sample.

Sequence analysis of the genes for Prn and the PT S1 subunit.

DNA sequencing of relevant regions of the *prn* and *s1* genes was performed on PCR fragments as described by Mooi *et al.* (1998). Sequence analyses of *prn* were confined to the two regions comprising 400 bases and encoding the repeats GGxxP and PQP, designated regions 1 and 2, respectively. Region 1 was sequenced in all strains. Since no polymorphism had been previously observed in region 2, this region was sequenced in one out of five strains. Since Prn5 was a new type, its gene was sequenced completely. The sequence revealed that polymorphism was restricted to region 1. The *s1* gene was sequenced completely. The S1 and Prn types of the Chiron and SKB vaccine strains (W165 and Tohama, respectively) were also determined. The origin of the strain used for the Connaught whole-cell vaccine was unknown. Therefore, the S1 and Prn types were determined with the vaccine preparation used in the field trial. After amplification, the PCR products were sequenced. Appropriate negative controls were used to monitor possible contamination.

DNA fingerprinting. Strains were fingerprinted by hybridization of *Sma*I-digested DNA with a probe derived from IS1002, essentially as described by van der Zee *et al.* (1996). Three to four external standards in each gel were used to facilitate comparison of fingerprint types between gels. After transfer to a membrane, hybridizing bands were detected with the Enhanced Chemiluminescence Gene Detection System (Amersham International). Exposed films were read with a scanner at 190 d.p.i. (HP Scanjet IIcx/T; Hewlett Packard).

Computer-assisted analysis of the IS1002 fingerprint patterns was carried out using GelCompar software (Windows NT version 4.1, Applied Maths, Kortrijk, Belgium).

Statistical analysis. *P* values (two-sided) were calculated with the chi-square test.

RESULTS

Polymorphism in Prn

Sequence analyses of *prn* were confined to the two regions encoding the repeats GGxxP and PQP, designated region 1 and region 2, respectively (Mooi *et al.*, 1998). Consistent with earlier observations only polymorphism in region 1 was found. Polymorphism in region 1 is probably generated by insertion or deletion of a repeat unit encoding the (closely related) repeats

Prn1	<u>RGD</u>	APA	GGAVP	GGAVP	GGAVP	GGFGP	GGFGP	-----	VL
Prn2	<u>RGD</u>	APA	GGAVP	GGAVP	GGFGP	GGFGP	GGFGP	GGFGP	VL
Prn3	<u>RGD</u>	APA	GGAVP	GGAVP	GGFGP	GGFGP	GGFGP	-----	VL
Prn5	<u>RGD</u>	APA	GGAVP	GGFGP	GGFGP	GGFGP	-----	-----	VL

Fig. 1. Amino acid sequence variants of Prn observed in *B. pertussis* strains isolated in Italy during the trial for the efficacy evaluation of acellular pertussis vaccines. In Prn2, Prn3 and Prn5, differences with respect to Prn1 are indicated in bold. The RGD sequence involved in adherence to host tissues is underlined.

GGAVP and GGFGP. Four Prn variants were found in the 129 strains analysed in this study, designated Prn1, Prn2, Prn3 and Prn5 (Fig. 1). Prn1, Prn2 and Prn3 have been described previously (Mooi *et al.*, 1998), whereas Prn5 represents a novel type.

Interestingly, in one of the strains the size of the PCR product indicated that an insertion had occurred in region 2. DNA sequencing revealed that a copy of the insertion sequence element IS481, which is present in approximately 80 copies in the *B. pertussis* chromosome (MacLafferty *et al.*, 1988), was inserted into region 2 of the *prn* gene. The size of the IS element was consistent with the increase in size of the PCR fragment.

The Chiron and SKB vaccine strains contained Prn1. The Connaught vaccine preparation contained DNA

encoding a novel Prn type. This type was identical to Prn1 in regions 1 and 2, except for a single non-silent mutation outside the region encoding the PQP repeats (not shown).

Table 1 shows the distribution of the Prn variants among the strains isolated in the four different groups of the Italian trial. Prn3 was the most prevalent, being present in 51% of all strains tested, followed by Prn2 (41%). Prn1 was detected in 6% of strains examined. Interestingly, six of the eight Prn1 strains had been isolated from unvaccinated children. The newly described Prn5 seems to be rare (2%). Prn2 and Prn3 were equally distributed between the two acellular vaccine groups, whereas in the whole-cell vaccine group the Prn3 was predominant over Prn2 (63 versus 33%). The differences between distribution of variants among the three vaccine groups and the unvaccinated group were not significant. However, when the vaccinated groups were pooled an interesting difference, although not statistically significant, in the Prn1 frequency could be observed compared to the unvaccinated children (13 and 2%, respectively; $P=0.08$).

Table 2, showing the Prn variant distribution among regions, indicates that most Prn1 strains (five out of eight) were isolated in Puglia, the region with a low vaccination coverage (41%) against pertussis during the years before the trial. Interestingly, in the same region,

Table 1. Distribution of Prn variants among 129 *B. pertussis* strains isolated in different vaccine groups of the Italian clinical trial

Values are the number of strains, with the percentage in parentheses.

Prn variant	Vaccine group				Total (%)
	Unvaccinated	Chiron	SKB	Connaught	
1	6 (13)	2 (7)	0	0	8 (6)
2	17 (35)	12 (45)	16 (53)	8 (33)	53 (41)
3	23 (48)	13 (48)	14 (47)	15 (63)	65 (51)
5	2 (4)	0	0	1 (4)	3 (2)
Total	48 (37)	27 (21)	30 (23)	24 (19)	129 (100)

Table 2. Distribution of Prn variants among 129 *B. pertussis* strains isolated in different Italian regions participating in the clinical trial

Values are the number of strains, with the percentage in parentheses.

Prn variant	Region				Total (%)
	Friuli	Veneto	Piemonte	Puglia	
1	0	1 (3)	2 (5)	5 (10)	8 (6)
2	4 (100)	10 (27)	15 (41)	24 (47)	53 (41)
3	0	25 (67)	20 (54)	20 (39)	65 (51)
5	0	1 (3)	0	2 (4)	3 (2)
Total	4 (3)	37 (28)	37 (28)	51 (41)	129 (100)

Fig. 2. Normalized DNA fingerprint types found in strains isolated during the Italian field trial. The numbers on the left refer to the sizes of DNA markers.

Prn3, predominant in other regions, was exceeded by Prn2.

The addition of another 24 strains from Puglia to the initial sample of 105 to further search for other strains with Prn1 or Prn5 only confirmed the results already

obtained, although this caused a final imbalance in the proportion of strains among the various groups.

IS1002-associated RFLP in *B. pertussis* strains

To investigate the genetic relationship among the *B. pertussis* isolates characterized by the presence of different Prn variants, 76 out of the 129 strains studied for the Prn polymorphism were analysed for IS1002-associated RFLP.

The analysis was performed on all the strains with the newly described Prn5, on most strains with Prn1, and on a similar number of Prn2 and Prn3 randomly chosen, ensuring that more than 50% of strains with a specific Prn type were analysed.

Fourteen different fingerprint types were observed (Fig. 2). Three fingerprint types were found to predominate, 29, 12 and 35, which represented 41, 23 and 11% of the strains, respectively (Table 3). These three fingerprint types also predominate in the Netherlands (unpublished data). The Prn1 strains belonged to the RFLP types 12 and 29. Strains with Prn3 belong to five different RFLP types with a predominance of types 12 and 29. Strains with Prn2 belonged to 12 different types with a predominance of types 29 and 35. Many unique fingerprint types were found in the Prn2 group. The three strains with Prn5 showed the same fingerprint type 10. Interestingly, fingerprint type 35 was detected only among Prn2 strains, fingerprint type 12 predominated in strains with Prn3 and fingerprint type 29 was equally distributed among strains with different Prn variants. The differences in fingerprint frequencies between Prn2 and Prn3 strains were significant ($P=0.002$). The number of Prn1 and Prn5 strains which were fingerprinted

Table 3. Fingerprint types in relation to the Prn variants in 76 *B. pertussis* strains isolated during the Italian clinical trial

Fingerprint type	Prn variant				Total (%)
	1	2	3	5	
7	0	1	0	0	1 (1)
9	0	1	0	0	1 (1)
10	0	1	0	3	4 (5)
12	3	1	13	0	17 (23)
19	0	2	0	0	2 (3)
26	0	2	0	0	2 (3)
28	0	0	1	0	1 (1)
29	2	13	16	0	31 (41)
35	0	8	0	0	8 (11)
55	0	1	2	0	3 (4)
63	0	3	0	0	3 (4)
70	0	1	0	0	1 (1)
71	0	1	0	0	1 (1)
72	0	0	1	0	1 (1)
No. of strains (%)	5 (7)	35 (46)	33 (43)	3 (4)	76 (100)
No. of fingerprint types	2	12	5	1	14

Table 4. Distribution of different fingerprint types in 76 *B. pertussis* strains isolated from vaccinated and unvaccinated children participating in the Italian clinical trial

Values are the number of strains, with the percentage in parentheses.

Fingerprint type	Vaccinated	Unvaccinated
7	1 (2)	—
9	1 (2)	—
10	2 (5)	2 (7)
12	11 (25)	6 (19)
19	1 (2)	1 (3)
26	1 (2)	1 (3)
28	1 (2)	—
29	19 (42)	12 (39)
35	3 (7)	5 (16)
55	—	3 (10)
63	3 (7)	—
70	1 (2)	—
71	1 (2)	—
72	—	1 (3)
No. of strains (%)	45 (100)	31 (100)
No. of fingerprint types	12	8

was too low for meaningful analysis. As far as differences between strains from vaccinated and unvaccinated children are concerned (Table 4), types 12 and 29 are predominant in both groups (67 and 58%, respectively) while type 35 seems to be mainly present in the latter (16%), although numbers are again too low for meaningful analysis. A difference in the types was also observed since only types 10, 12, 19, 26, 29 and 35 are present in both groups.

Geographical distribution is shown in Table 5. Fingerprint type 29 showed a more or less equal distribution among the regions. The frequency of fingerprint type 12 was much higher in Veneto (34%) than in Piemonte and Puglia (18 and 16%, respectively). For the other fingerprint types showing unequal distributions, including type 35, the number of strains involved was

quite low and the significance of these findings remains to be substantiated. When the analysis was limited to the three dominant fingerprint types (12, 29 and 35), only differences between Puglia and Veneto were found to be significant ($P = 0.044$).

PT S1 subunit polymorphism

Thirty *B. pertussis* strains (19 from vaccinated and 11 from unvaccinated children) were analysed for polymorphism in the *s1* gene. All the strains showed sequences of the *s1* gene corresponding to the *s1A* variant, also observed in the majority of the recent Dutch isolates of *B. pertussis* (Mooi *et al.*, 1998). The Chiron and SKB vaccine strains contained S1B, while the Connaught vaccine preparation contained DNA encoding S1D.

DISCUSSION

The recent literature (Andrews *et al.*, 1997; Bass & Wittler, 1994; de Melker *et al.*, 1997; De Serres *et al.*, 1995) has documented the increase in the incidence of pertussis in highly vaccinated populations, and this has led to the hypothesis that *B. pertussis* strains have evolved which are less affected by vaccine-induced immunity (Mooi *et al.*, 1998; Weiss & Hewlett, 1986). Indeed, in a previous study of the Dutch *B. pertussis* population it was found that antigenic shifts had occurred in two proteins important for immunity, Prn and PT, resulting in antigenic divergence between vaccine strains and clinical isolates (Mooi *et al.*, 1998). The availability of strains collected during the recent Italian field trial (Greco *et al.*, 1996; Mastrantonio *et al.*, 1995) provided a unique opportunity to compare strains isolated from vaccinated and unvaccinated children, and from regions which had different vaccine coverages during the years before the beginning of the Italian trial.

In Italy, most of the children were not vaccinated against pertussis for the fear of side effects until the beginning of the double-blind clinical trial (Greco *et al.*, 1996) and the registration of the two acellular vaccines in 1995. It is noteworthy, however, that the previous vaccination policy differed from region to region, resulting in areas with higher coverage, such as the Veneto, Piemonte and

Table 5. Fingerprint types of 76 *B. pertussis* strains isolated in the four Italian regions participating in the clinical trial

Values are the number of strains, with the percentage in parentheses.

Region	Fingerprint type														Total
	7	9	10	12	19	26	28	29	35	55	63	70	71	72	
Friuli	0	0	0	0	0	0	0	1 (25)	3 (75)	0	0	0	0	0	4
Piemonte	0	0	0	2 (18)	0	1 (9)	0	6 (55)	0	0	0	1 (9)	0	1 (9)	11
Puglia	1 (3)	0	3 (10)	5 (16)	1 (3)	1 (3)	1 (3)	14 (45)	2 (7)	0	3 (10)	0	0	0	31
Veneto	0	1 (3)	1 (3)	10 (34)	1 (3)	0	0	10 (34)	3 (10)	3 (10)	0	0	1 (3)	0	30
Total	1 (1)	1 (1)	4 (5)	17 (23)	2 (3)	2 (3)	1 (1)	31 (41)	8 (11)	3 (4)	3 (4)	1 (1)	1 (1)	1 (1)	76

Friuli regions, and areas with a lower coverage, such as Puglia. However, figures for vaccination coverage in those years have been published only for Veneto and Puglia, respectively, 81 and 41 % (Cinquetti *et al.*, 1994; Lo Palco *et al.*, 1995).

The results of this study showed the circulation of four different Prn variants. Besides the three Prn variants found in the Dutch *B. pertussis* population, Prn1, Prn2 and Prn3 (Mooi *et al.*, 1998), a novel type, Prn5, was observed. The Prn1 variant is contained in the two acellular vaccines while the whole-cell DTP vaccine used in the field trial contained a Prn type which differed from Prn1 in one amino acid. Further, it seems likely that the Prn1 variant is also present in the whole-cell vaccines introduced in Italy in the 1950s and used previous to the field trial. This assumption is based on the observation that 25 strains, originating from a number of different countries (i.e. France, USA, UK, Japan, Finland, China and Australia) and isolated before 1960, all contained the Prn1 variant (F. R. Mooi, unpublished data). Among the Italian strains analysed, the frequency of Prn1 was low compared to Prn2 and Prn3 (6, 41 and 51%, respectively). The Prn5 variant was only observed in three strains, suggesting it may have emerged recently. It is interesting that, in contrast to Prn2 and Prn3, the frequency of Prn1 was lower in the vaccinated group compared to the unvaccinated group (2 versus 13%). Moreover the Prn1 variant was detected mainly (i.e. five out of eight Prn1 strains) in Puglia, the region with a low vaccination coverage during the years before the trial. These observations suggest that strains with Prn1 are more affected by vaccine-induced immunity than strains with Prn2 or Prn3 as already hypothesized in the Dutch study (Mooi *et al.*, 1998).

Strains with Prn3 were observed at a higher frequency in regions with a high vaccination coverage previous to the trial (Veneto and Piemonte, Prn3 frequencies 67 and 54%, respectively) compared to the region with low coverage (Puglia, Prn3 frequency 39%). In the group vaccinated with the Connaught whole-cell vaccine during the field trial, the frequency of Prn3 was nearly two times higher than the Prn2 frequency (63 and 33%, respectively). Although not statistically significant these data taken together suggest that vaccination with whole-cell vaccines is less effective against Prn3 strains compared to Prn2 strains. There was no evidence for different efficacies of acellular vaccines against strains with Prn2 or Prn3, as the frequencies of these Prn types were similar in the groups vaccinated with acellular vaccines.

Four PT S1 types (S1A–D) have been described (Mooi *et al.*, 1998), of which only S1A was found in the Italian strains. A similar observation was made within the Dutch *B. pertussis* population, although S1B was also detected at a low frequency (Mooi *et al.*, 1998). We found that the acellular and whole-cell vaccines used during the trial contained S1B or S1D. Thus, both with respect to Prn and PT, the Italian clinical isolates showed distinct variants when compared to vaccine strains. It is

important to note that, notwithstanding these differences between clinical isolates and vaccine strains, the acellular vaccines have been shown to be efficacious in the field trial (Greco *et al.*, 1996).

IS1002-based fingerprinting revealed three dominant fingerprint types (12, 29 and 35) that comprised 74 % of the strains. The same three types were found to predominate in the Dutch *B. pertussis* population (van Loo *et al.*, 1999). It is possible that these types may have spread because they are less affected by vaccine-induced immunity.

The distribution of the three dominant fingerprint types within the four Prn variants showed types 12 and 29 equally represented in the five analysed strains with the Prn1 variant (60 and 40%, respectively) and in those with the Prn3 variant (36 and 47%, respectively). In the Prn2 strains, type 29 predominated (37%), followed by type 35 (24%) and, at a very lower frequency, by type 12 (3%). Interestingly, in the Prn2 group, a greater number of different fingerprint types was observed compared to the Prn3 strains. In fact in 35 Prn2 strains we detected 12 fingerprint types whereas in 33 Prn3 strains only five types were observed. This is consistent with the observation made on a larger sample of Prn3 strains collected in the Netherlands, and it suggests that Prn3 strains are genetically less diverse than Prn2 and may have evolved more recently (van Loo *et al.*, 1999). The populations consisting of Prn2 and Prn3 strains were significantly different with respect to the frequency of fingerprint types ($P = 0.002$), suggesting distinct lineages. Thus it seems unlikely that strains switch their Prn type by insertion or deletion of repeats with a high frequency.

In conclusion, these data suggest that despite the fact that the vaccine coverage in Italy has been lower than in the Netherlands, the population structure of *B. pertussis* in the two countries was quite similar. This may be taken as evidence that the *B. pertussis* population structure was not affected by vaccination. Alternatively, the population structure may have been influenced by import from neighbouring countries with a high vaccine coverage. Finally, it is conceivable that even a relatively low vaccine coverage, like the one in Italy before the trial, has a significant effect on the bacterial population. The assumption that vaccination affects the *B. pertussis* population structure is consistent with the observation that vaccination selects against distinct strains. For example, strains with Prn1 (the vaccine type) are isolated with a lower frequency from vaccinated individuals compared to unvaccinated individuals (this study; Mooi *et al.*, 1998). Further, the Prn1 variant was detected mainly (i.e. five out of eight Prn1 strains) in Puglia, the region with a low vaccination coverage during the years before the trial. Recently it has been shown that the presence of antibodies against Prn and PT correlate with clinical protection (Cherry *et al.*, 1998; Storsaeter *et al.*, 1998). The circulation of variants in these antigens, which are part of most acellular vaccines, should be carefully monitored in view of their possible influence on vaccine efficacy.

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REFERENCES

- Ad Hoc Group for the Study of Pertussis Vaccines (1988).** Placebo controlled trial of two acellular pertussis vaccines in Sweden: protective efficacy and adverse events. *Lancet* **i**, 960–995.
- Andrews, R., Herceq, A. & Roberts, C. (1997).** Pertussis notifications in Australia. *Commun Dis Intell* **21**, 145–148.
- Bass, J. W. & Wittler, R. R. (1994).** Return of epidemic pertussis in the United States. *Pediatr Infect Dis J* **13**, 343–345.
- Cahill, E. S., O'Hagan, D. T., Illum, L. & Redhead, K. (1993).** Mice are protected against *Bordetella pertussis* infection by intranasal immunization with filamentous haemagglutinin. *FEMS Microbiol Lett* **107**, 211–216.
- Cherry, J. D., Gornbein, J., Heininger, U. & Stehr, K. (1998).** A search for serologic correlates of immunity to *Bordetella pertussis* cough illnesses. *Vaccine* **16**, 1901–1906.
- Cinquetti, S., Pupo, A., Carpenè, A., Salmaso, P., Pisani, F., Fosco, A. & Paganino, C. (1994).** Le malattie infettive nel Veneto 1991–1993. ed. Regione Veneto-Giunta Regionale.
- De Serres, G., Bouliane, N., Douville-Fradet, M. & Duval, B. (1995).** Pertussis in Quebec: ongoing epidemic since the late 1980s. *Can Commun Dis Rep* **15**, 45–48.
- Edwards, K. M. (1993).** Acellular pertussis vaccines: a solution to the pertussis problem? *J Infect Dis* **168**, 15–20.
- Greco, D., Salmaso, S., Mastrantonio, P. & 9 other authors (1996).** A controlled trial of two acellular vaccines and one whole cell vaccine against pertussis. *N Engl J Med* **334**, 341–348.
- Kimura, M. & Kuno-Sakai, H. (1990).** Developments in pertussis immunization in Japan. *Lancet* **336**, 30–32.
- Leininger, E., Keminer, J. G. & Brennan, M. J. (1990).** Surface proteins of *Bordetella pertussis*: role in adherence. In *Proceedings of the Sixth International Symposium on Pertussis*, pp. 100–105. Edited by C. R. Manclark. DHHS publication no. (FDA) 90-1164. Bethesda: US Public Health Service, Department of Health and Human Services.
- Leininger, E., Roberts, M., Kenimer, J. G., Charles, I. G., Fairweather, N., Novotny, P. & Brennan, M. J. (1991).** Pertactin, an Arg-Gly-Asp-containing *Bordetella pertussis* surface protein that promotes adherence of mammalian cells. *Proc Natl Acad Sci USA* **88**, 345–349.
- van Loo, I. H. M., van der Heide, H. G. J., Nagelkerke, N. J. D., Verhoef, J. & Mooi, F. R. (1999).** Temporal trends in the population structure of *Bordetella pertussis* in the years 1949–1996 in a highly vaccinated population. *J Infect Dis* **179**, 915–923.
- Lo Palco, P., Germinario, G., Quarto, M. & Barbuti, S. (1995).** Vaccinazioni obbligatorie e facoltative dell'infanzia: stima della copertura vaccinale in Puglia. *Ann Igien* **7**, 401–408.
- MacLafferty, M. A., Marcus, D. R. & Hewlett, E. L. (1988).** Nucleotide sequence and characterization of a repetitive DNA element from the genome of *Bordetella pertussis* with characteristics of an insertion sequence. *J Gen Microbiol* **134**, 2297–2306.
- Mastrantonio, P., Giuliano, M., Salmaso, S., Greco, D. & Wassilak, S. (1995).** The Italian trial of acellular pertussis vaccines. *Microecol Ther* **25**, 293–296.
- de Melker, H. E., Conyn-van Spaendonck, M. A. E., Rumke, H. C., van Wijngaarden, J. K., Mooi, F. R. & Schellekens, J. F. P. (1997).** Pertussis in The Netherlands: an outbreak despite high levels of immunization with whole cell vaccine. *Emerg Infect Dis* **3**, 175–178.
- Mooi, F. R., van Oirschot, H., Heuvelman, K., van der Heide, H. G. J., Gaastra, W. & Willems, R. J. L. (1998).** Polymorphism in the *Bordetella pertussis* virulence factors P.69/pertactin and pertussis toxin in The Netherlands: temporal trends and evidence for vaccine-driven evolution. *Infect Immun* **66**, 670–675.
- Peppoloni, S., Pizza, M., De Magistris, M. T., Bartoloni, A. & Rappuoli, R. (1995).** Acellular pertussis vaccine composed of genetically inactivated pertussis toxin. *Physiol Chem Phys Med NMR* **27**, 335–361.
- Relman, D. A., Domenighini, M., Tuomanen, E., Rappuoli, R. & Falkow, S. (1989).** Filamentous hemagglutinin of *Bordetella pertussis*: nucleotide sequence and crucial role in adherence. *Proc Natl Acad Sci USA* **86**, 2637–2641.
- Salmaso, S., Mastrantonio, P., Wassilak, S. G. F., Giuliano, M., Anemona, A., Giammanco, A., Tozzi, A. E., Ciofi degli Atti, M. L. & Greco, D. (1998).** Persistence of protection through 33 months of age provided by immunization in infancy with two three-component acellular pertussis vaccines. Stage II Working Group. *Vaccine* **13**, 1270–1278.
- Shahin, R. D., Brennan, M. J., Li, Z. M., Meade, B. D. & Manclark, C. R. (1990).** Characterization of the protective capacity and immunogenicity of the 69-kD outer membrane protein of *Bordetella pertussis*. *J Exp Med* **171**, 63–73.
- Storsaeter, J., Hallander, H. O., Gustafsson, L. & Olin, P. (1998).** Levels of anti-pertussis antibodies related to protection after household exposure to *Bordetella pertussis*. *Vaccine* **16**, 1907–1916.
- Trollfors, B., Taranger, J., Lagergard, T., Lind, L., Sundh, V., Zackrisson, G., Lowe, C. U., Blackwelder, W. & Robbins, J. B. (1995).** A placebo-controlled trial of pertussis-toxoid vaccine. *N Engl J Med* **333**, 1045–1050.
- Weiss, A. A. & Hewlett, E. L. (1986).** Virulence factors of *Bordetella pertussis*. *Annu Rev Microbiol* **40**, 661–686.
- Willems, R. J. L. & Mooi, F. R. (1996).** From whole cell to acellular pertussis vaccines. *Rev Med Microbiol* **7**, 13–21.
- van der Zee, A., Vernooij, S., Peeters, M., van Embden, J. D. A. & Mooi, F. R. (1996).** Dynamics of the population structure of *Bordetella pertussis* as measured by IS1002-associated RFLP: comparison of pre- and post-vaccination strains and global distribution. *Microbiology* **142**, 3479–3485.

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