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Antigenic Divergence of *Bordetella pertussis* Isolates in Taiwan

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In recent studies, antigenic divergence has been observed in *Bordetella pertussis* circulating isolates. We collected 80 *Bordetella pertussis* isolates in Taiwan from 1998 to 2004 and analyzed them using a combination of pulsed-field gel electrophoresis (PFGE) and sequencing of the *ptxS1* and *prn* genes. The incidence of pertussis increases every 3 years, and most of the isolates prevalent since 1998 have expressed nonvaccine *ptxS1A* and *prn2* alleles. Through PFGE analysis, all isolates could be classified into four major groups, and the incidence of these groups exhibited a correlation with the *prn* allele expressed by the isolates. We found that PFGE is more discriminative than gene sequencing, since it could divide the isolates expressing the *prn2* allele into two groups: one group circulating from 1998 to 2001 and another group circulating from 2001 to 2004. The transition between the two groups in 2000 coincided with an outbreak of 326 cases. This research indicates that the antigenic divergence of *B. pertussis* circulating isolates has evolved over time in Taiwan. Such information will have implications for vaccine policy in Taiwan.

Pertussis, known as whooping cough, is a highly transmissible disease of the respiratory system caused by *Bordetella pertussis.* Before vaccination, pertussis was one of the major causes of death of infants and children. It is presently one of the 10 most common causes of death from infectious diseases worldwide (10). In the 1950s, whole-cell pertussis vaccines (WCVs) were introduced in many countries and have been effective in reducing the incidence of pertussis (10, 21). In Taiwan, diphtheria-pertussis-tetanus (DPT) vaccines have been offered since 1954, and they successfully decreased the incidence of reported pertussis cases from 691 in 1955 to less than 10 in 1970 (3, 12). In 1996, acellular pertussis vaccines (ACVs), combined with diphtheria and tetanus toxoids as DTaP, were introduced. Currently, two acellular pertussis vaccines are in use in Taiwan, and they now constitute more than 50% of pertussis vaccine usage in Taiwan.

The low incidence of pertussis was retained from 1971 to 1991, but then an outbreak of 226 reported cases occurred in 1992. This unexpected reemergence of pertussis also occurred in other countries with high rates of vaccination coverage with WCV, such as The Netherlands (5, 16), the United States (3), and Finland (15). With the advent of molecular epidemiology, it has been suggested that such a resurgence can be attributed to the antigenic divergence between vaccine strains and circulating strains (8, 11, 17).

Pulsed-field gel electrophoresis (PFGE) and gene sequencing analysis are two standard technologies which have been used in epidemiological studies of *B. pertussis* isolates (14). PFGE is highly reproducible and stable and provides a genome-wide perspective for monitoring outbreaks of *B. pertussis* (1, 2, 7, 19). Gene sequencing analysis applies to genes with specified relevance, such as virulence determinants. Pertussis toxin (PT) and pertactin (Prn) are two major virulence factors of *B. pertussis* and are constituents of most ACVs; hence, it is significant to study the antigenic divergence in these proteins. Antigenic shifts in the genes encoding the S1 subunit of PT (*ptxS1*) and Prn (*prn*) between vaccine strains and circulating strains have been demonstrated, and it is suggested that these shifts were caused by vaccination (6, 15, 23).

Temporal changes in the types of *B. pertussis* clinical isolates have been observed in most developed countries (24, 25). In this study, 80 *B. pertussis* isolates dating from the period from 1998 to 2004 were collected in Taiwan, and the antigenic divergence of the clinical isolates was investigated by a combination of PFGE analysis and gene sequencing of the *ptxS1* and *prn* alleles. Such long-term molecular surveillance data can contribute not only to vaccine choice and production but also to the consolidation of vaccine policy.

MATERIALS AND METHODS

Isolates. A total of 80 *B. pertussis* strains isolated from 1998 to 2004, collected from different parts of Taiwan, were used in this study. All of the isolates were confirmed to be *B. pertussis* by Gram staining, oxidase tests, and slide agglutination tests with *B. pertussis* antiserum (Difco, BBL). *B. pertussis* strains were grown on Bordet-Gengou agar supplemented with 15% defibrinated horse blood at 37°C for 4 to 5 days.

PFGE analysis. Strains were inoculated on Bordet-Gengou agar (Difco) for 96 h at 37°C. Colonies on agar were picked in a cell suspension buffer (100 mM Tris-HCl, 100 mM EDTA, pH 8.0). The cell density of the suspension for the plug was estimated by using a turbidity meter (VITEK Colormeter) and adjusting the turbidity to 15%. A total of 400 μl of the cell suspension was transferred to a 1.5-ml microcentrifuge tube, and 20 μl of proteinase K (20 mg/ml; Sigma) was added. Then, 400 μl of 1% (wt/vol) agarose (Seakem Gold agarose; BioWhittaker Molecular Applications) in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) was added. After the components were mixed, the solution was poured into the wells of plug molds and kept at room temperature for 5 min for solidification. The plugs were transferred to 50-ml tubes containing 5 ml of cell lysis buffer (100 mM Tris-HCl, 0.45 M EDTA, pH 8.0, 1% *N*- lauroyl sarcosine, 1 mg/ml proteinase K) and incubated for 2 h in a shaker water bath at 54°C. The plugs were washed twice, first with double-distilled H_2O at 54°C for 15 min and then with TE buffer at 54°C for 10 min. They were then stored in TE buffer until use. For restriction enzyme digestion, the plug slices were transferred to 200 μl

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TABLE 1. Number of pertactin and pertussis toxin S1 subunit sequence types of 80 *B. pertussis* isolates in Taiwan from 1998 to 2004

Yr isolated	No. of isolates	No. of isolates				
		Pertactin			Pertussis toxin S1 subunit	
		1	2	3	A	B
1998	9	3	6	0	9	0
1999	10	2	7	1	9	1
2000	22	0	21	1	22	0
2001	5	0	5	0	5	0
2002	9	0	9	0	9	0
2003	16	1	15	0	16	0
2004	9	0	9	0	9	0
Total	80	6	72	2	79	1

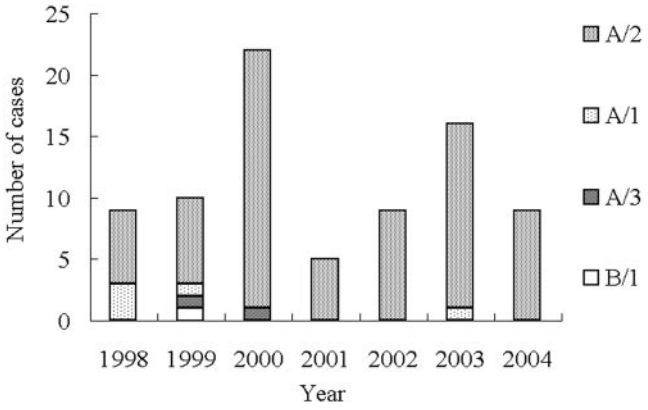

FIG. 1. Temporal trends in frequencies of pertactin (1 to 3) and pertussis toxin (A or B) types of 80 *B. pertussis* isolates in Taiwan.

of a buffer solution containing 10 U of XbaI (Promega) and incubated at 37°C for 2 h. The cleaved DNA fragments were separated by using a CHEF-DRIII apparatus (Bio-Rad), with pulse times ranging from 4 to 40 s, at an angle of 120° at 14°C and 6 V/cm in 1% SeaKem Gold agarose gel with 0.5× TBE (Tris-borate-EDTA) for 19 h. After electrophoresis, the gel was stained with ethidium bromide solution and destained with distilled water. DNA fragments were imaged with an IS-1000 digital imaging system (Alpha Innotech) (14).

Analysis of banding patterns. Dendrogram analysis was performed by using Bionumerics software, version 3.0 (Applied Maths). The position tolerance was set at 0.8%, and optimization was set at 1%. The Dice coefficient was used to analyze the similarities (S_{AB} values) of the band patterns. The unweighted pair group method with arithmetic averages (UPGMA) was used for cluster analysis. Isolates were considered different when the band similarity value was less than 95%.

PCR amplification. The total genomic DNA of the strain was extracted by using the PUREGENE DNA purification kit (Gentra, Minneapolis, Minn.), as described previously (9). The concentration of DNA extracted from the *B. pertussis* isolates was measured with a spectrophotometer (A_{260}). The DNA was stored at −80°C until use. The primers used to target the pertussis toxin S1 subunit (*ptxS1*) and pertactin (*prn*) genes were those described previously (14). The conditions for amplification of the *ptxS1* and *prn* genes were as follows: amplification of the genes was conducted in 50 μl of a mixture containing 40 ng template DNA, 5% dimethyl sulfoxide, 200 μM each deoxynucleotide, 10 pmol of each primer, and 2× PCR master mix (Fermentas). The reaction mixtures were preheated at 95°C for 5 min and then underwent 30 amplification cycles that consisted of the following program: 20 s at 95°C, 30 s at 59°C, and 1 min at 72°C. The final extension was for 10 min at 72°C.

DNA sequencing. The PCR products were purified with a QIAquick PCR purification kit (QIAGEN), and both strands were sequenced with the primers used for amplification as described previously (14). The sequencing reactions were carried out with a BigDye Terminator kit, and the products were analyzed on an ABI 377 DNA sequencer (Applied Biosystems).

RESULTS

Polymorphism in the pertactin and pertussis toxin S1 subunit. The *ptxS1* and *prn* genes of all 80 *B. pertussis* isolates collected during the period from 1998 to 2004 were sequenced. Four *ptxS1* and eight *prn* variant types have been described previously (14). Only two pertussis toxin S1 subunit alleles (*ptxS1A* and *ptxS1B*) and three pertactin alleles (*prn1*, *prn2*, and *prn3*) were found in the 80 Taiwanese isolates (Table 1). From 1998 to 2004, the *ptxS1A* and *prn2* alleles were prevalent and identified in 79 (99%) and 72 (90%) of 80 isolates, respectively. For pertactin there was a shift in predominance. In 1998, *prn1* was present in one-third of the isolates. In 1999, the ratio of *prn1* decreased to 20%. Later, in 2003, only one isolate with *prn1* was identified. The *tcfA* genes, which encode the tracheal colonization factor, from 80 isolates were sequenced between bases 255 and 698. No polymorphism was found.

Correlation between pertactin and pertussis toxin types. Two *ptxS1* alleles and three *prn* alleles were identified. A total of six theoretical combinations exist. However, only four types were observed in Taiwan isolates: *ptxS1B*/*prn1* (B/1), old; *ptxS1A*/*prn1* (A/1), transitional; and *ptxS1A*/*prn2* (A/2) and *ptxS1A*/*prn3* (A/3), new, similar to the findings in other countries (11, 19).

As shown in Fig. 1, of the 80 isolates, 72 (90%) with new *ptxS1A*/*prn2* alleles were prevalent strains and 5 (6%) isolates had the transitional *ptxS1A*/*prn2* alleles from 1998 to 2004. It is interesting that only one isolate was found to have the old type, *ptxS1B*/*prn1* (B/1), and that only two isolates had the new type, *ptxS1A*/*prn3* (A/3) (11).

PFGE typing. The PFGE patterns of the 80 isolates were examined. Nineteen PFGE profiles were identified (Fig. 2). The 19 profiles were classified into four major groups; group I included one profile, group II comprised three profiles, group IIIa included six profiles, and group IIIb had nine profiles. However, 74 (93%) of the 80 isolates belonged to groups IIIa and IIIb and comprised the major group of isolates.

Trend of *ptxS1A*/*prn2* type in Taiwan. The distribution of the isolates of four PFGE groups according to the year of collection is shown in Fig. 3. Groups IIIa and IIIb containing the *ptxS1A*/*prn2* type were clearly prevalent from 1998 to 2004. Few isolates of groups I and II were circulating before 1998.

The bottom panel of Fig. 4 shows the distribution of the isolates of groups IIIa and IIIb according to the year of collection. All isolates in 1998 exhibited characteristics of group IIIa, and then the number of isolates in group IIIa decreased, whereas the number of isolates of group IIIb increased in the later years. The change of the prevalent strain was observed during the period from 1998 to 2002, and an outbreak in 2000 coincided with the transition of PFGE groups IIIa and IIIb (Fig. 4, top).

DISCUSSION

Recent reports have described a reemergence of pertussis in many countries with high vaccination rates, including Australia, the United States, and The Netherlands (5, 7, 20).

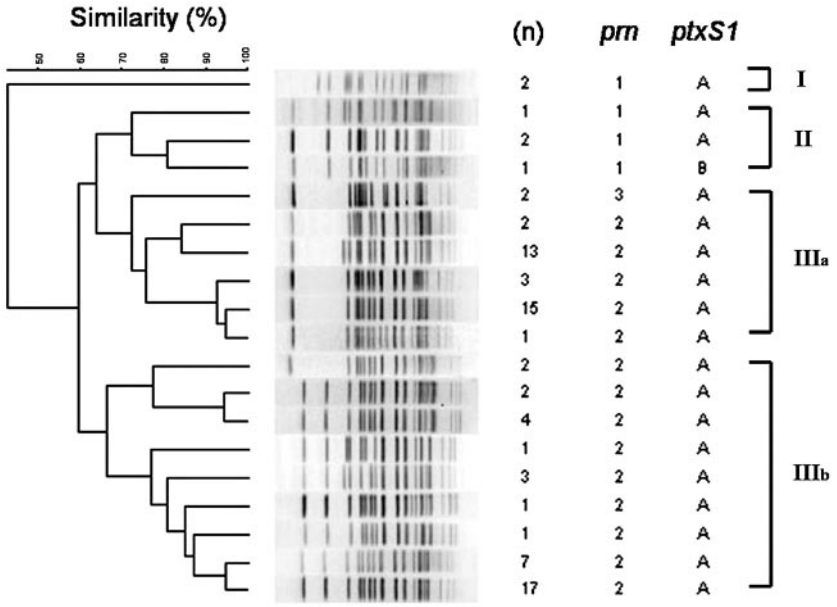

FIG. 2. Classification of 80 *B. pertussis* isolates in Taiwan by PFGE. The dendrogram was prepared by using UPGMA. *ptxS1*/*prn*, the combination of *ptxS1* and *prn* alleles; n, number of isolates.

Such a phenomenon may have been caused by decreases in vaccine coverage, improved diagnostic methods, and changes in vaccine quality. These possibilities have been excluded for The Netherlands (4), and antigenic divergence between the vaccine strain and circulating isolates in The Netherlands is consistent with the notion of vaccine-driven evolution (16). In France, The Netherlands, Finland, and the United States, nonvaccine types of *ptxS1A*/*prn2* (A/2) gradually replaced the vaccine types *ptxS1B*/*prn1* (B/1) (3, 5, 15, 25). We investigated the antigenic divergence in 80 Taiwan *B. pertussis* isolates from 1998 to 2004 and found that nonvaccine *prn2* and *ptxS1A* alleles were identified in 72 (90%) of 80 isolates.

In Taiwan, despite the high rate of coverage of the vaccine, cases of pertussis have still been reported each year and the incidence of *B. pertussis* has peaked every 3 years (Fig. 1). WCV, produced by Pasteur-Mérieux (now Aventis-Pasteur), has been in use since 1954. WCV is procured by the govern-

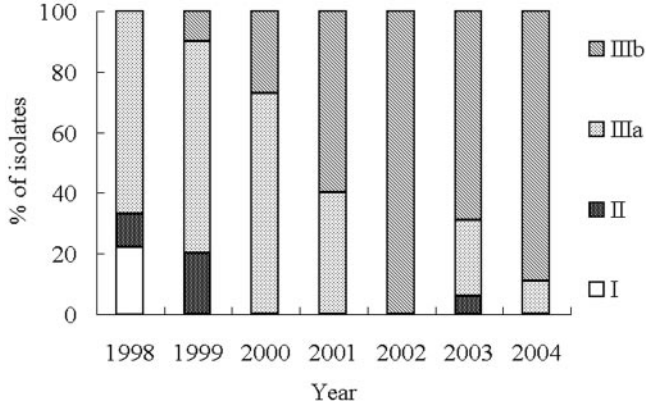

FIG. 3. Distribution of isolates of PFGE groups I, II, IIIa, and IIIb according to the year of collection.

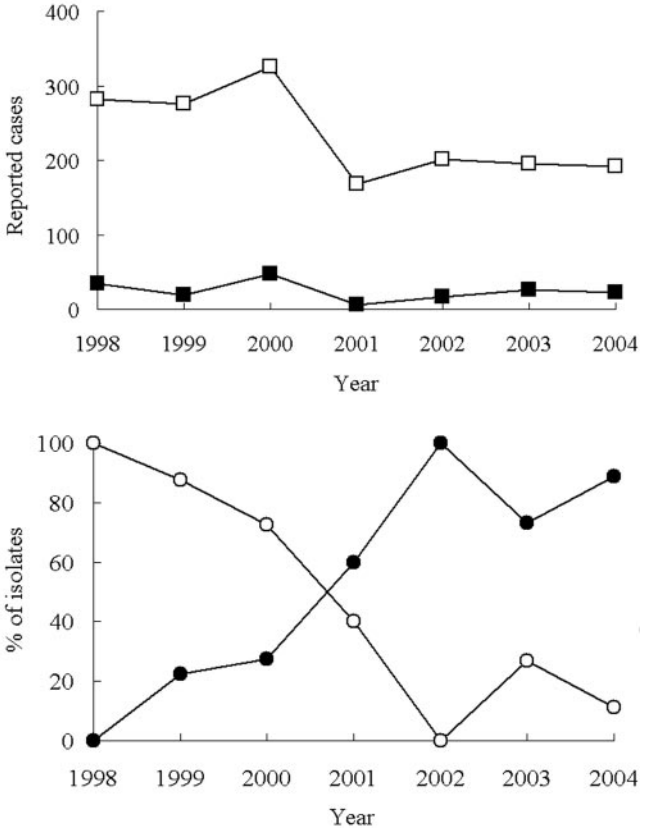

FIG. 4. Temporal trends in reported pertussis cases and isolates of PFGE group IIIa and IIIb in Taiwan. (Top) Pertussis-like cases (□) and pertussis cases (■) reported from different hospitals in Taiwan from 1998 to 2004. The data were compiled from the Center for Disease Control of Taiwan. (Bottom) Changes in the frequencies of PFGE group IIIa (○) and IIIb (●) according to the year of collection.

ment and is offered free of charge. This vaccine, produced from a mixture of the IM1414 and the IM1416 strains, has the *prn1* allele and the two *ptxS1* alleles *ptxS1B* and *ptxS1D*, as described previously (18). Besides WCV, acellular pertussis vaccines, from Kaketsuken during 1996 and 1997 and from 1998 on from SmithKline Beecham Biologicals, both of which contain the *ptxS1B*/*prn1* type, were also gradually introduced (3, 11). ACV is offered at the patient's own expense. However, due to the low rate of adverse reactions of ACV, it is gaining acceptance and the rate of usage is increasing. The evolution of nonvaccine type *ptxS1A*/*prn2* (A/2) in Taiwan is very similar to its evolution in France, which has also had a vaccination history similar to Taiwan's with respect to the types and strains used in the vaccine and vaccination schedules (22). Another two countries, Finland and The Netherlands, have used WCVs exclusively since 1952 and 1953, respectively, and also show a similar tendency for the nonvaccine type to be responsible for more than 90% of cases between 1990 and 1996 (15, 17). Interestingly, the neighboring Asian country of Japan, which used WCV beginning in since 1950 and ACV since 1981, had a 78% incidence of vaccine type *ptxS1B*/*prn1* (B/1), only a 17% incidence of nonvaccine type *ptxS1A*/*prn2* (A/2), and a 4% incidence of transitional type *ptxS1A*/*prn1* (A/1) from 1988 to 2001. It was postulated that the nonvaccine type *ptxS1A*/*prn2* (A/2) may have recently been imported into Japan (11). Japan was the first country to use ACV exclusively, and it has been in use for more 20 years, 15 years ahead of Taiwan. Whether the different antigenic divergence pattern between Japan and Taiwan is due to such vaccine policy differences deserves further investigation.

In previous reports, no high degree of correlation between the PFGE type and the combination of *ptxS1* and *prn* alleles was found in Canadian and U.S. *B. pertussis* isolates (3, 25). In Japan, there was a correlation between the PFGE types and the *ptxS1*/*prn* alleles (11). However, in France it was reported that the PFGE type correlated with the *prn* allele but not with the *ptxS1* allele (25). Among Taiwanese isolates, most of the isolates (97%) in groups IIIa and IIIb contained a nonvaccine *prn2* allele, while 100% of group I and II isolates had a vaccine *prn1* allele (Fig. 2). However, no distinct correlation between the PFGE type and the *ptxS1* allele was found, as only one vaccine *ptxS1B* allele was found in 1999, but there may be a correlation with the year. The isolates collected before vaccination expressed *ptxS1B* or *ptxS1D*, whereas the isolates collected after 1991 expressed a new *ptxS1A* allele, as observed in other countries (3, 17). In Taiwan, from 1998 to 2004, most of the isolates collected (90%) contained a *ptxS1A*/*prn2* allele. The *ptxS1A*/*prn2* isolates can be divided into two further groups by using PFGE, one group that circulated from 1998 to 2001 and one group that circulated from 2001 to 2004, and are not associated with the expression of the new *prn* or *ptxS1* allele (Fig. 2). It is suggested that PFGE is more discriminatory than gene sequence typing on the basis of the characteristics of these *B. pertussis* isolates. During the period from 1998 to 2002, the epidemic *B. pertussis* isolates experienced a shift between PFGE group IIIa and group IIIb, and this evolution of *B. pertussis* isolates was not reflected in antigenic changes (Fig. 4). Group IIIa seemed to evolve in 1995, and its prevalence gradually increased. In 1997, there was a larger outbreak with 491 reported cases with a predominance of group IIIa (41%) (13).

The antigenic divergence between recent clinical isolates and the vaccine strains has been observed since 1998. It was shown that the PFGE type revealed a correlation with the *prn* allele but had no high correlation with the *ptxS1* allele and that all *tcfA* alleles exhibited the same type. Genes other than these three should also be investigated. Our results also suggest that the nonvaccine *ptxS1A*/*prn2* isolates have undergone continuous evolution in Taiwan. The finding that a transition between the PFGE groups in 2000 coincided with an outbreak of 326 cases highlights the importance of the study of circulating strains of *B. pertussis* in Taiwan. Such data will be helpful for the consolidation of vaccine policy in Taiwan.

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REFERENCES

1. **Advani, A., D. Donnelly, and H. Hallander.** 2004. Reference system for characterization of *Bordetella pertussis* pulsed-field gel electrophoresis profiles. J. Clin. Microbiol. **42:**2890–2897.
2. **Bisgard, K. M., C. D. Christie, S. F. Reising, G. N. Sanden, P. K. Cassiday, C. Gomersall, W. A. Wattigney, N. E. Roberts, and P. M. Strebel.** 2001. Molecular epidemiology of *Bordetella pertussis* by pulsed-field gel electrophoresis profile: Cincinnati, 1989–1996. J. Infect. Dis. **183:**1360–1367.
3. **Cassiday, P., G. Sanden, K. Heuvelman, F. Mooi, K. M. Bisgard, and T. Popovic.** 2000. Polymorphism in Bordetella pertussis pertactin and pertussis toxin virulence factors in the United States, 1935–1999. J. Infect. Dis. **182:** 1402–1408.
4. **de Melker, H. E., M. A. Conyn-van Spaendonck, H. C. Rumke, J. K. van Wijngaarden, F. R. Mooi, and J. F. Schellekens.** 1997. Pertussis in The Netherlands: an outbreak despite high levels of immunization with whole-cell vaccine. Emerg. Infect. Dis. **3:**175–178.
5. **de Melker, H. E., J. F. Schellekens, S. E. Neppelenbroek, F. R. Mooi, H. C. Rumke, and M. A. Conyn-van Spaendonck.** 2000. Reemergence of pertussis in the highly vaccinated population of The Netherlands: observations on surveillance data. Emerg. Infect. Dis. **6:**348–357.
6. **Fry, N. K., S. Neal, T. G. Harrison, E. Miller, R. Matthews, and R. C. George.** 2001. Genotypic variation in the *Bordetella pertussis* virulence factors pertactin and pertussis toxin in historical and recent clinical isolates in the United Kingdom. Infect. Immun. **69:**5520–5528.
7. **Hardwick, T. H., P. Cassiday, R. S. Weyant, K. M. Bisgard, and G. N. Sanden.** 2002. Changes in predominance and diversity of genomic subtypes of Bordetella pertussis isolated in the United States, 1935 to 1999. Emerg. Infect. Dis. **8:**44–49.
8. **He, Q., J. Makinen, G. Berbers, F. R. Mooi, M. K. Viljanen, H. Arvilommi, and J. Mertsola.** 2003. Bordetella pertussis protein pertactin induces type-specific antibodies: one possible explanation for the emergence of antigenic variants? J. Infect. Dis. **187:**1200–1205.
9. **Hsu, M. C., K. W. Chen, H. J. Lo, Y. C. Chen, M. H. Liao, Y. H. Lin, and S. Y. Li.** 2003. Species identification of medically important fungi by use of real-time LightCycler PCR. J. Med. Microbiol. **52:**1071–1076.
10. **Kerr, J. R., and R. R. Matthews.** 2000. Bordetella pertussis infection: pathogenesis, diagnosis, management, and the role of protective immunity. Eur. J. Clin. Microbiol. Infect. Dis. **19:**77–88.
11. **Kodama, A., K. Kamachi, Y. Horiuchi, T. Konda, and Y. Arakawa.** 2004. Antigenic divergence suggested by correlation between antigenic variation and pulsed-field gel electrophoresis profiles of *Bordetella pertussis* isolates in Japan. J. Clin. Microbiol. **42:**5453–5457.
12. **Lai, H. K., and S. F. Tsai.** 2000. Epidemiology of pertussis in Taiwan, 1993–1998, and its control measures. Epidemiol. Bull. **16:**255–281.
13. **Lee, Y. S., C. Y. Yang, C. H. Lu, and Y. H. Tseng.** 2003. Molecular epidemiology of Bordetella pertussis isolated in Taiwan, 1992–1997. Microbiol. Immunol. **47:**903–909.
14. **Mooi, F. R., H. Hallander, C. H. Wirsing von Konig, B. Hoet, and N. Guiso.** 2000. Epidemiological typing of Bordetella pertussis isolates: recommendations for a standard methodology. Eur. J. Clin. Microbiol. Infect. Dis. **19:** 174–181.
15. **Mooi, F. R., Q. He, H. van Oirschot, and J. Mertsola.** 1999. Variation in the *Bordetella pertussis* virulence factors pertussis toxin and pertactin in vaccine strains and clinical isolates in Finland. Infect. Immun. **67:**3133–3134.

16. **Mooi, F. R., I. H. van Loo, and A. J. King.** 2001. Adaptation of Bordetella pertussis to vaccination: a cause for its reemergence? Emerg. Infect. Dis. **7:**526–528.
17. **Mooi, F. R., H. van Oirschot, K. Heuvelman, H. G. van der Heide, W. Gaastra, and R. J. Willems.** 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.
18. **Njamkepo, E., F. Rimlinger, S. Thiberge, and N. Guiso.** 2002. Thirty-five years' experience with the whole-cell pertussis vaccine in France: vaccine strains analysis and immunogenicity. Vaccine **20:**1290–1294.
19. **Peppler, M. S., S. Kuny, A. Nevesinjac, C. Rogers, Y. R. de Moissac, K. Knowles, M. Lorange, G. De Serres, and J. Talbot.** 2003. Strain variation among *Bordetella pertussis* isolates from Quebec and Alberta provinces of Canada from 1985 to 1994. J. Clin. Microbiol. **41:**3344–3347.
20. **Poynten, M., P. B. McIntyre, F. R. Mooi, K. J. Heuvelman, and G. L. Gilbert.** 2004. Temporal trends in circulating Bordetella pertussis strains in Australia. Epidemiol. Infect. **132:**185–193.
21. **Robinson, A., L. I. Irons, and L. A. Ashworth.** 1985. Pertussis vaccine: present status and future prospects. Vaccine **3:**11–22.
22. **van Amersfoorth, S. C., L. M. Schouls, H. G. van der Heide, A. Advani, H. O. Hallander, K. Bondeson, C. H. von Konig, M. Riffelmann, C. Vahrenholz, N. Guiso, V. Caro, E. Njamkepo, Q. He, J. Mertsola, and F. R. Mooi.** 2005. Analysis of *Bordetella pertussis* populations in European countries with different vaccination policies. J. Clin. Microbiol. **43:**2837–2843.
23. **van Loo, I. H., K. J. Heuvelman, A. J. King, and F. R. Mooi.** 2002. Multilocus sequence typing of *Bordetella pertussis* based on surface protein genes. J. Clin. Microbiol. **40:**1994–2001.
24. **van Loo, I. H., H. G. van der Heide, N. J. Nagelkerke, J. Verhoef, and F. R. Mooi.** 1999. Temporal trends in the population structure of Bordetella pertussis during 1949–1996 in a highly vaccinated population. J. Infect. Dis. **179:**915–923.
25. **Weber, C., C. Boursaux-Eude, G. Coralie, V. Caro, and N. Guiso.** 2001. Polymorphism of *Bordetella pertussis* isolates circulating for the last 10 years in France, where a single effective whole-cell vaccine has been used for more than 30 years. J. Clin. Microbiol. **39:**4396–4403.