Molecular epidemiology of *Bordetella* spp.

Name of researcher: Shu-Man Yao, Chuen-Sheue Chiang
Affiliation: Centers for Disease Control, Ministry of Health and Welfare, Taipei, Taiwan

Summary:

Our government provides free vaccination for disease prevention when facing the threat of pertussis. Whole-cell pertussis vaccines (WCVs) have been replaced by acellular pertussis vaccines (ACVs) in many countries with vaccination policy. In Taiwan, free ACVs have been provided since 2010. Under selection pressure of vaccination, *Bordetella pertussis* tend to change its antigen genetics for vaccine components, from the genotype of vaccine strain (*ptxA2/prn1* or *ptxA4/prn1*) to the genotype of non-vaccine strain (*ptxA1/prn2* or *ptxA1/prn3*), from *ptxP1* to *ptxP3* to produce more virulent factors in order to fight immunity. Although pertactin-deficient (PRN-) bacteria resulted in disease epidemics in many countries, only a few sporadic strains were discovered in Taiwan. Majority of *B. pertussis* strains express pertactin.

I. Purpose:

Under selection pressure of vaccination, in addition to altering genetics for *ptxA*, *prn*, *fim3* and *ptxP* genes, *B. pertussis* strains that do not express pertactin are found recently in Japan, France, Finland and the USA. First pertactin-deficient (PRN-) *B. pertussis* strain was identified in Japan in 1997. Among 121 *B. pertussis* strains collected between 1990 and 2009, 27% strains did not express pertactin. In Japan, ACVs have been used for vaccination since 1981, and pertactin-deficient (PRN-) *B. pertussis* strains began to increase since early 2000 (1). In France, ACVs have been used since 1998 and completely replaced WCVs in 2002. Pertactin-deficient (PRN-) *B. pertussis* strains began to appear 7 years after ACVs usage. In 2007, 7.8% *B. pertussis* strains were pertactin-deficient whereas 13.3% *B. pertussis* strains were pertactin-deficient in 2011 (2). In Finland, ACVs completely replaced WCVs in 2005, and 2 pertactin-deficient (PRN-) *B. pertussis* strains were identified in 2011 (3). In the USA, 11 pertactin-deficient (PRN-) *B. pertussis* strains were identified in 2011-2012 (4). Pertactin is one component of some ACVs, playing a role in inducing immune protection. It is believed that appearance of pertactin-deficient (PRN-) strains is the result of *B. pertussis* adaption to selection pressure of vaccination.

In Taiwan, ACVs have been in the market since 1996, first at the patients' expense, then provided free by our government since 2010. Under selection pressure of vaccination, it is very much concerned whether genetics and gene expression of *B.*

pertussis strains will be altered, and whether effectiveness of vaccine protection will be affected.

II. Methods:

1. Analysis of genetic polymorphism for important *B. pertussis* antigen genes: DNA fragments for polymorphic regions of *ptxA*, *prn*, *fim3* and *ptxP* genes will be produced by suitable PCR primers, and DNA sequence will be obtained and analyzed (5-7).
2. Analysis of pertactin expression in *B. pertussis* strains by Western blotting: Protein will be extracted from cultured *B. pertussis* strains, quantitated, separated on SDS-PAGE, and transferred to nitrocellulose membrane. Pertactin is then detected by Western blotting using luminescenct system (1).
3. Analysis of fimbriae serotype for *B. pertussis* strains by glass agglutination: The WHO International Standard Monoclonal Antibodies, NIBSC 06/124and NIBSC 06/128, will be used for serotyping *B. pertussis* fimbrial antigen 2 and 3, respectively. Freshly cultured (72 hours) *B. pertussis* will be suspended in normal saline and then mixed with 1:10 diluted monoclonal antibodies. The agglutination reaction result will be observed after 1 minute (5).

III. Results:

Polymorphism of *B. pertussis ptxA*, *prn*, *fim3* and *ptxP* genes

Analysis of genetic polymorphism for *ptxA*, *prn*, *fim3* and *ptxP* genes was applied to 354 *B. pertussis* strains collected in 1992-2013. Three types of *ptxA* gene were detected, including *ptxA1*, *ptxA2* and *ptxA5*. Since 1993, the major type is *ptxA1*. *ptxA5* only appeared in 1992 and *ptxA2* only appeared in 1999. Six types of *prn* gene were detected, including *prn1*, *prn2*, *prn3*, *prn4*, *prn6* and *prn7*. Before 1997, the major type was *prn1*. Since 1998, *prn2* became dominant and consisted of more than 90% strains after 2000. Three types of *ptxP* gene were detected, including *ptxP1*, *ptxP3* and *ptxP4*. It is known that pertussis toxin and pertactin expression increase in strains that carry *ptxP3* gene. In 1995, *ptxP3* appeared and quickly became dominant, being detected in 44.4% strains in 1998, in 66.7% strains in 1999, and more than 85% since 2000. Three types of *fim3* gene were detected, including *fim3-1*, *fim3-2* and *fim3-4*. *fim3-1* was dominant before 1998 and started to decline in 1999. The proportion of *fim3-1* was 88.9%, 66.7%, 40%, 28.6%, 33.3%, 8.3% and 17.4% in 1999-2005, respectively. No *fim3-1* was detected in 2006. However, the proportion of *fim3-1* started to increase again, 7.1%, 9.5%, 20% and 15.4% in 2007-2010, respectively. The proportion of *fim3-1* reached 82.1%, and maintained at more than 80% thereafter.

Pertactin and fimbriae expression in *B. pertussis* strains

Expression of pertactin and fimbriae among 241 *B. pertussis* strains collected in 2003-2014 was analyzed and shown in the table. Two strains were pertactin-deficient, in 2011 and 2014. Among all *B. pertussis* strains analyzed, 99.2% strains produced pertactin and 0.8% did not produce pertactin. Among *B. pertussis* strains, 97.3% express serotype 3 fimbriae (Fim3) and 3.7% express serotype 2 fimbriae (Fim2). One strain expressed both Fim2 and Fim3. One strain did not express either Fim2 or Fim3.

	2003	2004	2005	2006	2007	2008	2009	2010	2011	2012	2013	2014
no	15	11	23	15	28	21	30	13	28	24	16	17
PRN+	15	11	23	15	28	21	30	13	27	24	16	16
PRN-	0	0	0	0	0	0	0	0	1	0	0	1
Fim2 +	1	0	1	0	1	2	0	0	1	0	0	1
Fim3 +	14	10	22	15	27	19	30	13	26	24	16	16
Fim2+Fim3+	0	1	0	0	0	0	0	0	0	0	0	0
Fim2- Fim3-	0	0	0	0	0	0	0	0	1	0	0	0

IV. Discussion:

In the investigation of global evolution of *B. pertussis* (8), genetic variations were observed for important *B. pertussis* antigen genes during 4 periods that is defined to reflect worldwide changes in pertussis vaccination, the early WCV period (earlier than 1960), the period in which mainly WCVs were used (WCV 1960-1995), the period in which both WCVs and ACVs were used (WCV/ACV 1996-2000), and a period in which mainly ACVs were used (ACV period, later than 2000). The most frequently observed *ptxA* genes were *ptxA1*, *ptxA2* and *ptxA4*. Both *ptxA2* and *ptxA4* dominated in the early WCV period (earlier than 1960). In 1921-1932, before the introduction of WCVs, *ptxA1* appeared and consisted of about 5% strains in the early WCV period, 68% in WCV 1960-1995, 92% in WCV/ACV 1996-2000 and 90% in ACV period (later than 2000). In Taiwan, *ptxA1* consisted of 91.7% strains in 1992-1996, and increased to 99.6% in 1997-2010 and 100% in 2011-2013, indicating that it was already under selection pressure in the WCV 1960-1995 period.

The most frequently observed *prn* genes were *prn1, prn2* and *prn3*. In the early WCV period, *prn1* dominated. In the WCV 1960-1995 period, *prn2* and *prn3* occurred, with *prn3* consisting of constant 10-17% and *prn2* increasing from 18% in the WCV 1960-1995 period to 65% in the ACV period (later than 2000). In Taiwan, *prn2* appeared in the WCV 1960-1995 period and consisted of 33% in 1992-1996, 85% in 1997-2010, and 96% in 2011-2013, again under strong selection pressure in the ACV period.

There are 5 types of *fim3*, with *fim3-1* (vaccine type) and *fim3-2* the major global types. The formal has been the major dominant type until the later was detected in the WCV 1960-1995 period when it consisted of 1% and continued to increase to 37% in

the ACV period. In Taiwan, the proportion of *fim3-1* was 97% in 1992-1996 and decreased to 39% in 1997-2010 whereas the proportion of *fim3-2* increased to 61%. In 2011-2013, the proportion of *fim3-1* and *fim3-2* reversed again, with *fim3-1* increasing to 85% whereas *fim3-2* decreasing to 13%. This result indicated that *fim3* gene was also under selection of host immune pressure to vary with time. However, different from *ptxA* and *prn* genes, *fim3* gene was likely less affected by passive vaccination and more affected by host immunity, thus, vaccine type and non-vaccine type alternate to dominate. Adaption to environment likely results in variations in the dominant strains, and in turn resulted in increasing prevalence of pertussis in Taiwan in 2009-2011.

There are more than 10 types for *ptxP* gene, with *ptxP1* and *ptxP3* most prevalent. In the early WCV period (earlier than 1960) and the WCV 1960-1995 period, *ptxP1* was dominant, consisting of 68% and 83%, respectively. Then, *ptxP3* began to replace *ptxP1*, consisting of 48% and 57% in the WCV/ACV 1996-2000 and ACV (later than 2000) period, respectively. The occurrence of *ptxP3* gene was in 1974-1977. The strains that carry *ptxP3* gene can produce more pertussis toxin (9), enabling strains to adapt to host immunity, and caused epdemics globally, e.g. in Canada, Sweden, the Netherlands, France, Australia, Japan and Korea since 1996 (7, 9-14). In Taiwan, *ptxP3* was first detected in 1995, consisted of 66.7% strains in 1999 and increased to more than 85% since 2000. Since 1997, the increased prevalence of pertussis in Taiwan is likely related to the dissemination of *ptxP3* strains globally.

Serotype of fimbriae was affected by introduction of vaccine. In England, use of WCVs resulted in reduction of Fim2/Fim3 or Fim2 strains (15). The proportion of Fim2 was 58% and Fim2/Fim3 was 13% in the prevaccine era (1920-1956), and Fim2 was 16% and Fim2/Fim3 was 38% in the early postvaccination era (1963-1967). In the mid-1970s, following the decline in pertussis vaccination in the UK due to concerns about vaccine safety, the proportion of Fim2 strains increased. Fim3 strains predominated again following the introduction of five component ACVs. However, Fim3 predominated in some countries where ACVs do not include Fim2/Fim3, indicating no direct relation to the usage of ACVs. In the study of antigen role played by fimbriae, passive immunity induced by vaccines had a stronger protection against Fim2 than Fim3 (16). It was suggested that antigenic difference, influenced by host acitve and passive immunity, enabled Fim3 strains to gain adaptation advantage. In Taiwan, Fim3 strains predominated (97.3%). Both Fim2 and Fim3 are important component in WCVs and ACVs, consideration of new vaccine component in future should be careful.

The appearance of pertactin-deficient (PRN-) strains caused pertussis epidemics in many countries (1-3, 17, 18). In mechanism investigation for the deficiency, most

cases are due to insertion of IS481 (insertion sequence 481) that affected gene expression, some cases are due to regulation of transcription or translation, but not gene mutations. Since genetic characteristics of pertactin-deficient (PRN-) *B. pertussis* strains are different in different countries, it was suggested not to be resulted from a clonal dissemination (18). Pertactin-deficient (PRN-) strains might avoid host ACVs-induced immunity, but how about pathogenesis? In vitro study revealed that pertactin-deficient (PRN-) strains grow faster than pertactin-proficient (PRN+) strains (1), thus, lack of pertactin expression did not affect transmission of strains. Severity of disease and length of hospitalization was no difference between pertactin-deficient (PRN-) strains and pertactin-proficient (PRN+) strains (19). In Taiwan, pertactin-proficient (PRN+) strains predominated and pertactin-deficient (PRN-) strains only occurred sporadically. Since pertactin is one component of ACVs, whether prevalence of pertactin-deficient (PRN-) strains will continue to increase in future should be closely monitored.

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VI. Publication list for this work:

Nil