中濃度が50μg/mL以上，MEPMでも26.9μg/mLに達する．MDRSPに対するそれらの薬剤のMICよりも血中濃度は上まわっており，臨床効果が認められたものと考えられる．ただし，化膿性髄膜炎や膿胸等を発症した場合には，上述した投与量では薬剤の移行が悪く，抗菌薬は恐らく無効で予後は不良となる可能性が高い[1]．また，ここに記したような症例においては，肺炎は治癒しても鼻咽頭の肺炎球菌は消失せずに残存している可能性がある．そして，介護施設へ戻ると，再びそれらを拡散させている可能性も否定できない．その他に，介護施設関係者や医療従事者を介しての拡散もあり得ることである．

結論として，肺炎球菌感染症に限らず，介護関連施設と治療目的の患者を受け入れる病院は，常に耐性菌情報を共有し，その拡散を未然に防止するための密接な連携が必要であることを強調しておきたい．

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利益相反自己申告：申告すべきものなし

文　献

1) 平成22年度厚生労働科学研究費補助金「新型インフルエンザ等新興・再興感染症研究事業」研究課題：重症型のレンサ球菌・肺炎球菌感染症に対するサーベイランスの構築と病因解析，その診断・治療に関する研究（研究代表　生方公子）（H22-新興-一般-013）．2012；p. 2—35.
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Genetic Analysis of Multidrug-resistant *Streptococcus pneumoniae* Including Meropenem Resistance that was Isolated from Elderly Residents with Pneumonia in Nursing-care Facilities

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From February to December 20XX, penicillin-resistant *Streptococcus pneumoniae* (PRSP) showing MICs of 16-32μg/mL to cefotaxime (CTX) and 4-8μg/mL to meropenem (MEPM) were isolated from 6 patients hospitalized at the general hospital S (2 cases) and hospital A (4 cases), close to the hospital S. Five elderly patients among these six cases came from nursing care facilities or nursing care-related medical facilities. All elderly persons (mean age : 81.7 years) were diagnosed as having pneumonia at the time of admission and the problematic PRSP was isolated from sputum samples collected on admission.

Notably, all of these PRSP isolates simultaneously showed high resistance to macrolide agents mediated by an *erm* (B) gene and to fluoroquinolone agents via mutations in the *gyrA* and *parC* genes. Eventually, they were identified as multidrug-resistant *S. pneumoniae* (MDRSP) with high resistance to many agents. The capsule type of all strains was serotype 19F and multilocus sequence typing (MLST) revealed that they belonged to clonal complex (CC) 7993, which has not been reported before. It was thus concluded that the MDRSP that had spread within the nursing facilities was transmitted to the general hospitals via the elderly inpatients with pneumonia caused by these agents. Although one case finally had a poor outcome, the pneumococcal infection was not the direct trigger of the event.

The current ratio of MDRSP is concluded to be very low. However, general hospitals that accept patients for therapeutic purposes from nursing-care facilities have to share epidemiological information in a timely manner with the nursing homes to prevent nosocomial infections.

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Investigation in a murine model of possible mechanisms of enhanced local reactions to post-primary diphtheria-tetanus toxoid boosters in recipients of acellular pertussis-diphtheria-tetanus vaccine

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Keywords: acellular pertussis vaccine, local reaction, histamine sensitizing activity, carbohydrate binding, enzymatic-HPLC assay

Abbreviations: aP, acellular pertussis vaccine; D, diphtheria; Dd, D toxoid; DTd, D and T combined toxoids; DTaP, acellular pertussis diphtheria-tetanus combined vaccine; DTwP, whole-cell pertussis vaccine combined with D and T toxoids; E-HPLC, enzyme coupled-high performance liquid chromatography; HS, histamine sensitizing; HIST, histamine sensitization test; Mab, monoclonal antibody; MFS, mouse footpad swelling; NIBSC, National Institute for Biological Standards and Control; pAb, polyclonal antibody; PT, pertussis toxin; PTd, pertussis toxoid; T, tetanus; wP, whole-cell pertussis vaccine

In recipients primed with acellular pertussis diphtheria-tetanus combined vaccine (DTaP) an increased incidence of severe local reactions with extensive redness/swelling has been reported for each subsequent dose of diphtheria-tetanus based combination vaccine given as a booster. This has been attributed to residual active pertussis toxin (PT) in the primary vaccine. In this study, we investigated the possible contribution of the A-subunit enzymatic activity and the B-oligomer carbohydrate binding activity of residual PT in DTaP to local reactions in a murine model using Japanese DTaP batches produced before and after the introduction of a test for reversion of pertussis toxoid to toxin. Residual PT activity was correlated with the B-oligomer carbohydrate binding activity. The in vivo mouse footpad swelling model assay indicated that the B-oligomer carbohydrate binding activity and possibly other factors were associated with intensified sensitization to local reaction following diphtheria toxoid booster.

Introduction

Acellular pertussis vaccine (aP) in combination with diphtheria (D) and tetanus (T) toxoids (DTaP) was developed successfully in Japan and has been used there since 1981.[1] It has proven to be clinically safe and effective and along with whole-cell pertussis vaccine (wP) combined with D and T toxoids (DTwP) is recommended by WHO for pediatric immunisation.[2-4] Although the rates of adverse reactions to DTaP are lower than for DTwP, DTaPs have been reported to cause occasional rather severe local reactions to booster doses of D and T combined toxoids (DTd, approximately one-fifth antigen content of the DTaP primary dose) given at 11–12 y of age in Japan.[5,6] The rates of local reactions increased with each subsequent booster dose of DT based combination vaccines and extensive local redness/swelling have also been reported for booster doses in DTaP-based schedules in other countries.[7-9]

In Japan in the 1980s it emerged that pertussis toxoid (PTd) in DTaP could revert to toxicity during long-term storage.[10] Accordingly, following extensive investigation of this issue, the detoxification process was improved by manufacturers and the levels of residual histamine sensitizing (HS) activity in Japanese DTaPs began to decline from 1985 onwards. This finally resulted in the revision of the Japanese Minimum Requirements for Biological Products[11] in 1991. The histamine sensitization test (HIST) for reversion of PTd to active pertussis toxin (PT) was implemented on DTaP incubated at 37 °C for 4 wk (accelerated reversion test) and also the specified upper limit for residual HS activity was revised from 0.8 to 0.4 HS units (HSU)/mL.[12] Subsequently, in a clinical surveillance study[13] performed during

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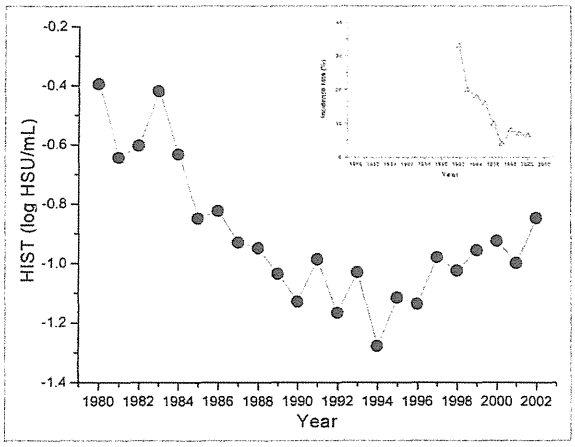

Figure 1. Change in annual mean residual HS activity of DTaP batches and local reactions to the booster dose with DTd. Annual geometric means of HS activity (●) of DTaP batches used for primary immunization were determined shortly after them being manufactured. Detoxification processes for aP antigens were revised from the mid-1980s without changing other specifications for DTaP. Local reactions following DTd booster in adolescents at the age of 11–12 y* were monitored from 1992 to 2000 in cohorts from Hisayama-cho, Fukuoka, Japan.[13] The number of adolescents (in brackets) who received the booster in these cohorts from 1992 to 2000 were 1992 (368), 1993 (986), 1994 (1170), 1995 (175), 1996 (150), 1997 (56), 1998 (310), 1999 (313), and 2000 (146). (△) is the percentage of local reaction incidence at the injection sites determined by size of area of redness of ≥5 cm in diameter observed 2 d after the booster; *The vaccination program in Japan was temporarily suspended due to 2 cases of severe adverse events after DTwP vaccination in 1975 and resumed 3 mo later to start at 2 y of age until revision of the immunization schedule in 1994 to start at 3 mo of age. Therefore all children in this surveillance study were immunized with DTaP at 2 y of age with the subsequent 3 doses at 3 to 8 wk intervals with an additional dose 12 to 18 mo later (primary immunization).

1992–2000 it was observed that the changes in annual mean residual HS activity in DTaP lots were associated with decreased annual incidence rates of local reactions following boosting with DTd in adolescents (**Fig. 1**). Thus, adolescents who received the primary DTaP immunizations before 1985 and were boosted with DTd in 1992–1993 showed a higher incidence of local reaction (measured as size of area of redness at the injection sites) in comparison with those receiving the DTaP primary immunizations after 1985 followed by the DTd booster after 1993.

PT is a member of the AB_5 family of bacterial toxins, having an A-subunit named S1, which is an ADP-ribosyltransferase that targets the α-subunit of some GTP-binding regulatory proteins.[14,15] The B-oligomer (B-subunit) of subunits S2 through to S5 is required for cell targeting and cytosolic entry of S1.[16,17] PT is an important protective antigen and in its detoxified form, PTd is included as a component in all types of aP vaccines. Although detoxified, the possibility of partial reversion to toxic PT activity of aP is recognized and monitoring for PT residual toxicity by the HIST is regarded as an essential part of the safety control of aP-containing vaccines and is required by regulatory authorities.[11,18,19]

Previous investigation performed in an animal model showed that excess residual active PT as indicated by residual HS activity in DTaP could cause intensified sensitization to subsequent severe local reaction to a booster dose of D toxoid (Dd) without augmenting IgG and IgE responses, suggesting a role for cell mediated immunity.[20] In the present study, to elucidate the role of subunits of PT in relation to local reactions, the activity of A- and B-subunits of PT in Japanese DTaP batches produced before and after 1990 were investigated using the mouse footpad swelling (MFS) model and established in vitro A- and B-subunits assay systems.[21-25]

Results

Relationship of ADP-ribosyltransferase activity to HS and sensitizing activities to MFS reaction

Eleven and 6 batches of DTaP produced before and after 1990, respectively, were assayed for enzymatic activity by enzyme coupled-high performance liquid chromatography (E-HPLC). They were also assayed for HS activity in mice and sensitizing activity to MFS and the results are represented in **Figure 2A and B**. Batch to batch variations in these assays were similar for vaccine batches made before and after 1990. Much reduced HS activities were seen in batches produced after 1990 in comparison with earlier ones. The difference between batches produced before and after 1990 was even more evident in the sensitizing activity to MFS compared with HS activity. Mean swelling ($\times 10^{-2}$ mm) for batches produced before 1990 was 57.2 ($\times 10^{-2}$mm) and that for batches produced after 1990 was 20.2 ($\times 10^{-2}$ mm) ($P < 0.0001$). However, similar levels of ADP-ribosyltransferase activity were observed for batches produced both before and after 1990, although considerable batch to batch variations were seen (**Fig. 2**). The results indicate that the enzymatic activity in those vaccines was not directly proportional to the observed decrease in both the HS activity and the sensitizing activity to MFS for the products manufactured after 1990. Furthermore, they also suggest that the change in the detoxification process after 1990 probably had a limited effect on the A-subunit of PT.

Carbohydrate binding activities of PT B-oligomer detected using different antibodies

Carbohydrate binding activities of PT B-oligomer using fetuin ligand in DTaP batches produced before and after 1990 were measured by detecting fetuin-bound PT using either a polyclonal antibody (pAb) against PT or monoclonal antibodies (Mabs) against S2&3 and S4 subunits of PT, respectively. Although large variations in binding activities were observed between these batches (**Fig. 3**), the overall results indicated that the vaccines produced after 1990 showed significantly lower binding activities than those produced before 1990 ($P < 0.05$) (**Fig. 3**).

In the HIST, the mean HS activity of batches produced before 1990 was 0.024 log HSU/mL and that of batches produced after 1990 was -0.767 log HSU/mL, an approximately 6-fold difference ($P = 0.0013$) (**Fig. 3**). In the fetuin-binding assay, all the antibodies detected carbohydrate binding activity

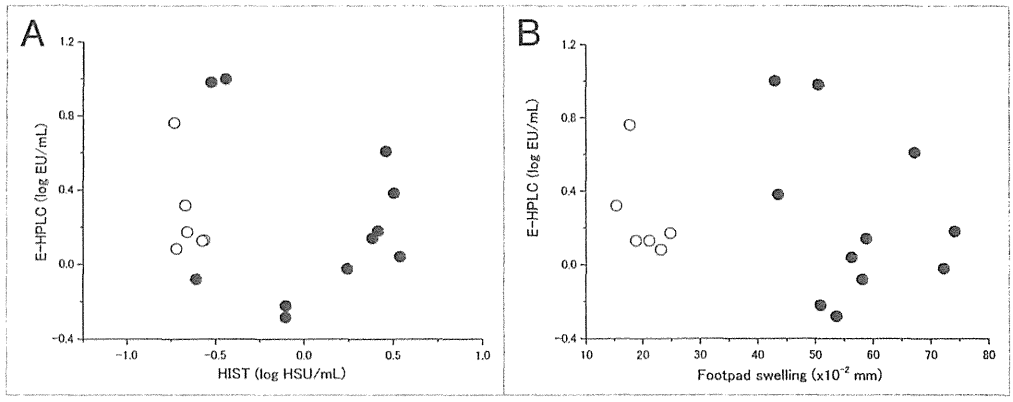

Figure 2. Relationship of residual enzymatic activity of S1 as measured by E-HPLC to (**A**) HIST and (**B**) sensitizing activity to mouse foot swelling (MFS) to Dd booster. Vaccine produced after 1990 differ to those before 1990 only in strengthened detoxification procedure for aP antigens and no change was made to other specifications. (●) DTaP (n = 11) produced before 1990; (○) DTaP (n = 6) produced after 1990.

differences between the 2 groups of DTaP vaccines, but the ratio of differences detected by Mab S4 was far lower than those detected using pAb or Mab S2&3. The ratio of difference in binding activities between vaccines produced before and after 1990 using different detecting antibody (**Fig. 3**) showed a ranking of Mab 2&3 (5.3) followed by pAb (4.1) and Mab S4 (2.6). This may be explained by the different efficiency of each detecting antibody e.g., they could show different abilities to distinguish between the 2 groups of vaccines on one hand, and on the other hand the changes to the detoxification procedure 1990 may have had greater impact on subunits 2&3 than subunit 4.

Carbohydrate binding activities and sensitizing activity to MFS of DTaP produced before and after 1990

DTaP batches produced before 1990 (11 batches) showed significant sensitization (>40 × 10^{-2} mm reaction) to MFS, while those produced after 1990 (6 batches) showed far less sensitization (<30 × 10^{-2} mm reaction) (**Fig. 4A–C**). Although no significant correlation could be seen between the sensitizing activities to MFS and carbohydrate binding activities using any of the detection antibodies, most vaccine batches produced before 1990 showed higher carbohydrate binding activity units (BU) with values between 1 BU/mL (0 log BU/mL) up to approximately 13 BU/mL (1.11 log BU/mL) and 65 BU/mL (1.81 log BU/mL) respectively when pAb and Mab 2&3 were used as detecting antibodies, while binding activities detected for most of the batches produced after 1990 were at or less than 0.76 BU/mL (-0.119 log BU/mL) except for one outlying batch (**Fig. 4A–C**). In the present study there was no significant sensitizing activity to MFS observed if residual B-subunit binding activity of PTd was below 0.76 BU/mL (–0.119 log BU/mL) (upper value detected by pAb except for an outlying value) or 1 BU/mL (0 log BU/mL) (upper value detected by Mab S2&3 except for an outlying value) detected by pAb or Mab S2&3, respectively. The results suggest that the higher carbohydrate binding activity of PTd in DTaP batches made before 1990 was related to the observed reactogenicity in the mouse model.

Discussion

In general, DTaPs have proved less reactogenic and safer than DTwPs.[1-3,26] However, severe local swelling has been regarded as a safety problem for booster immunizations with DTaP and DTd. Although the mechanisms of the reactogenicity remain unknown, residual PT activity of DTaPs for immunization was shown to play a role in the enhanced sensitization of mice to the DTd booster related hind paw swelling.[20] We attempted to investigate further the possible contribution of the enzymatic activity (A-subunit) and carbohydrate binding activity (B-oligomer) of PT to the enhanced local reaction in a mouse model.

Various chemical treatments have been used by manufacturers to detoxify PT. Although formaldehyde has been the only detoxification reagent used by all manufacturers in Japan,[27] different detoxification conditions e.g., formaldehyde concentration, incubation period, or temperature could result in different amino acid side-chain modifications and changes in conformational and linear epitope binding patterns for the resulting PTd.[28,29] This may reflect the variation in residual activities observed among these vaccines made both before and after 1990 from different manufacturers. Furthermore, although DTaP batches used in this study contained either aluminum hydroxide or aluminum phosphate (≤0.3mg Al/mL) according to manufacturer, these aluminum gels were reported not to be the major cause of injection site inflammation at the primary dose and also showed no sensitizing effect to DTd boosters.[20,30]

In general, it is noted that the detoxification procedure after 1990 had significant impact on B-subunit binding activity (**Figs. 3 and 4**) while the A-subunit enzymatic activity hardly changed (**Figs. 2 and 3**) when compared with earlier products. Therefore, the reduced residual PT activities observed in the in vivo assays for samples produced after 1990 were probably largely due to the reduction in B-subunit binding activities detected, whether by reduced binding to fetuin ligand or reduction/modification of epitopes recognized by the detection antibodies.

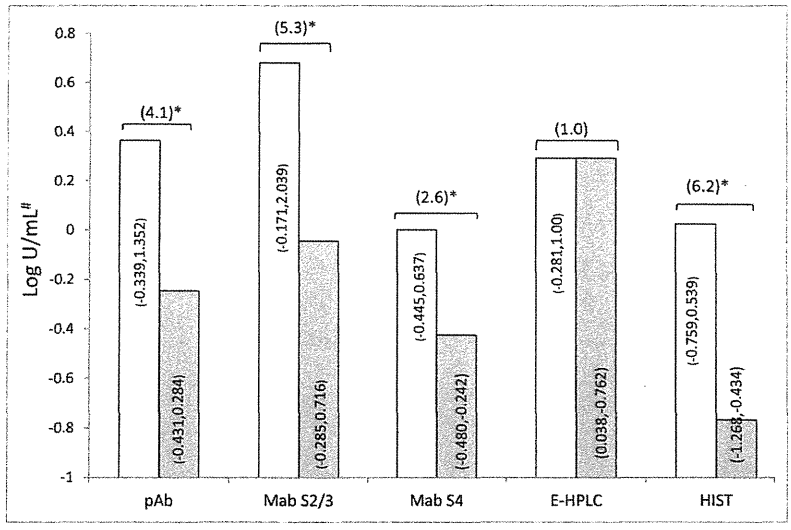

Figure 3. Comparison of carbohydrate binding, enzymatic and HS activities in DTaP made before (open bar, n = 11) and after (gray bar, n = 17) 1990. Values inside the bars represent the lowest and highest activities. Bracketed numbers outside of bars represent fold difference between before and after 1990. *Indicate statistically significant at 5% level. # log binding activity unit (BU/mL) for pAb, Mab S2&3, and Mab S4 binding assay; log enzymatic activity unit (EU/mL) for E-HPLC assay and log HSU/mL for HIST (see Methods Section).

Results from this study also suggest that the choice of detection antibody may impact on the detection efficiency of carbohydrate binding activity of chemically detoxified PTd. While the B-oligomer of PT has 4 distinct subunits, all amino acid residues involved in binding activities have thus far been mapped to the S2 and S3 subunits of the B-pentamer.[31-35] Multiple binding sites have been identified on S2 and S3 subunits.[36] In the present study, a pAb and different Mabs to different subunits in B-oligomer were used to detect the PT molecule bound to fetuin ligand. While there were no significant differences in binding results between pAb and Mab S2&3 as detection antibodies, significant differences were observed between binding activities assessed using Mab S2&3 and Mab S4 (P = 0.0007), or pAb and Mab S4 (P = 0.039) (**Fig. 3**). Although all the antibodies were able to detect a difference in binding activities between the samples produced before and after 1990, pAb and Mab S2&3 showed the biggest differential binding activities for products made before and after 1990 (**Fig. 3**). This lower sensitivity of Mab S4 in differentiating products made before and after 1990 suggests that the S4 epitope on the PTd molecule may have been less affected by the change in detoxification process after 1990 than the epitopes recognized by Mab S2&3. Since the exact mechanisms and sites of action of the different toxoiding reactions on the PT molecule have not yet been defined, using pAb as the detection antibody may be more appropriate for detecting possible structural changes by recognizing a wider range of epitopes on PTd molecules than a particular Mab. In any case, if a Mab is used in preference to pAb, Mab to subunits 2&3 would be more appropriate.

Although significant differences between the vaccines produced before and after 1990 were shown in residual HS activity and also in the sensitizing activity to MFS, enzymatic activity did not differ between these 2 groups. On the other hand, higher carbohydrate binding activities were seen for the vaccines produced before 1990 than those after 1990 and in most cases the binding activity showed a positive relationship to the sensitization activity to MFS.

Cell mediated immunity is reported to be a potential cause of local reactions to booster vaccination.[37] Although the precise mechanisms and the role of each A- or B-subunit for reactivity in vivo are unknown, B-subunit of PT was reported to enhance both Th1 and Th2 immune responses to co-administered antigens.[38] Our results suggest that B-oligomer binding activity may play a role in priming the intensification of the MFS and similarly the immune response intensified by B-subunit may result in intensified sensitization to booster doses. However, conclusive correlation of the binding activity to MFS was not established in the present study which suggests other unknown mechanisms via holotoxin activity could also play a role in the priming for intensified MFS to a co-administered antigen.[20] Although residual enzymatic activity of PT alone showed no direct relevance to the sensitization to MFS, the higher residual carbohydrate binding activity of PT could promote the entry of the A-subunit into cells in vivo and thus holotoxin activity could also contribute to the intensified MFS observed for the vaccines produced before 1990.

Materials and Methods

Materials

The PT used was a freeze-dried reference preparation (National Institute for Biological Standards and Control (NIBSC), 90/518).[39] Japanese reference pertussis vaccine for toxicity test lot 2 (reference vaccine) with a known HS activity (48 HSU/vial) was used as the reference preparation for HIST.

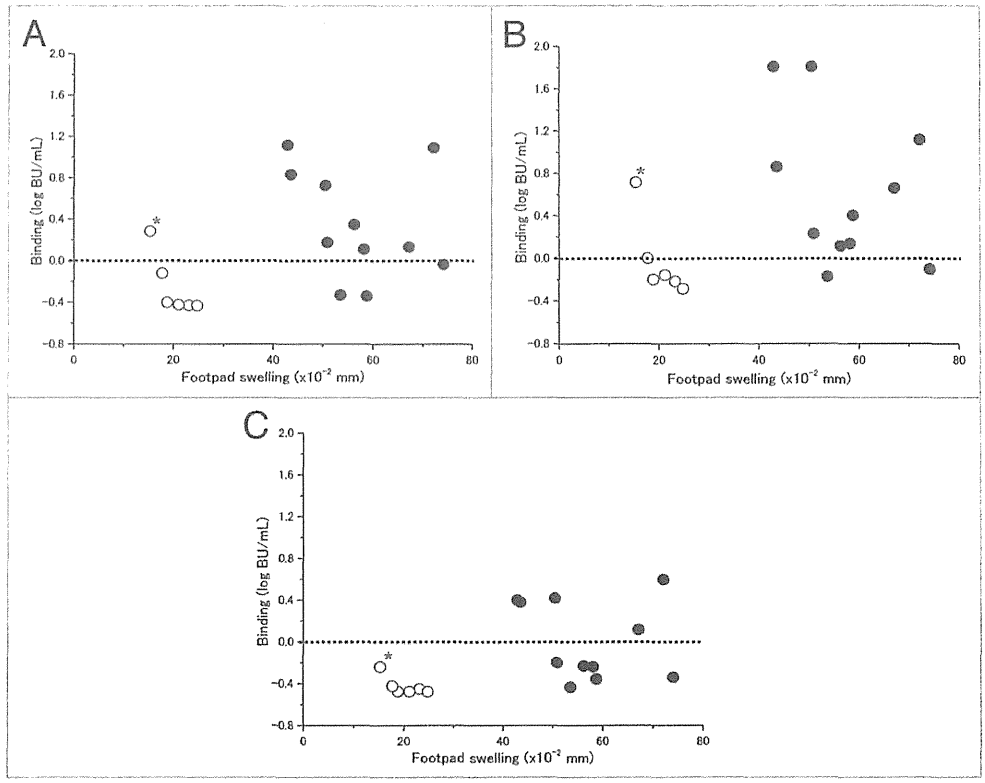

Figure 4. Relationships between sensitizing activity to footpad swelling and B-subunit carbohydrate binding activities to fetuin ligand detected using various antibodies: **(A)** pAb, **(B)** Mab S2&3, and **(C)** Mab S4. (●) DTaP (n = 11) produced before 1990; (○) DTaP (n = 6) produced after 1990 which differ from those produced before 1990 only in strengthened detoxification procedure for aP antigens. *Outlier (see Statistical analysis section under Materials and Methods).

A total of 28 batches of DTaP from 6 manufacturer sources in Japan were used in this study, which included vaccine batches produced in the 1980s (n = 11), before the implementation of a stricter regulation on HS activity for residual PT, and manufactured in the 1990s (n = 17), after the implementation of the regulation. Aluminum hydroxide or aluminum phosphate was contained as the adjuvant in the vaccines. While pertussis antigen formulations in these vaccines varied depending on the source manufacturer,[1,27] contents of D and T toxoids were almost the same for all the products. Those produced after 1990 differed from before 1990 only in strengthened detoxification procedure for pertussis antigens e.g., employing higher formalin concentration, longer period, or higher temperature of treatment etc. After obtaining, all vaccine samples were stored at 4–8 °C throughout.

The PT enzyme substrate, fluorescein-tagged Gαi3C20 peptide, F-VFDAVTDVIIKNNLKECGLY-COOH (F-Gαi3C20) was custom-synthesized by AnaSpec Inc. and was reported to have >95% purity. Polyclonal anti-PT antibody (pAb) (NIBSC, 97/572), monoclonal antibodies (Mabs) to S2&3 subunits (NIBSC, 99/534), and S4 subunits (NIBSC, 99/554) were from NIBSC. Peroxidase labeled anti-sheep IgG (A3415) and peroxidase labeled anti-mouse IgG (A0168) was obtained from Sigma. All other chemicals, unless specified otherwise, were of analytical grade and purchased from either Sigma-Aldrich or VWR-BDH.

E-HPLC coupled assay

The determination of ADP-ribosyltransferase activity of PT in vaccines was performed as previously described.[23,40] The enzyme activity was expressed as enzymatic activity units (EU) where 1 EU equals to fluorescence produced by 1 μg of PT (90/518) under identical assay and analysis conditions. All assays were performed in duplicate and met the in house assay validity criteria.[40]

Carbohydrate binding assay

The carbohydrate binding activity of PT in vaccines using bovine fetuin as carbohydrate coat was performed as described by Gomez et al.[24] Apart from polyclonal antibody (1/10000 dilution), Mab to S2&S3 and Mab to S4 were also used as the detecting antibody at 1/500 dilution. All samples were assayed in duplicate. PT (90/518) used in the assay is for calibration purpose for comparing differences between vaccine products in binding activity units (BU). BU is arbitrary units representing the binding activity in vaccine products. The potency of PT binding activity in vaccine relative to 90/518 was calculated using a parallel line analysis and was expressed as arbitrary binding activity units (BU) where 1 BU equals to optical density produced by 1 μg of PT (90/518) under identical assay and analysis conditions.

Temperature method of HIST

The HIST by temperature measurement was performed as described in previous publications.[11,41] The HS activity in the

vaccines was calculated relative to that of the reference vaccine using a parallel line analysis and was expressed as HSU.

Mouse footpad swelling (MFS) model

An animal model in which mice are primed with DTaP and boosted (challenged) with Dd was used to assess the sensitizing activity to intensified local reaction (swelling size) as described by Yamamoto et al.[20] In brief, BALB/c female mice were injected intramuscularly with an immunizing vaccine (DTaP) twice at a one-month interval. They were injected intraperitoneally with indomethacin daily from 3 d before challenge and their right and left hind paws were injected subcutaneously with a 50 μL dose of Dd (diluted to 30 Lf/mL with saline containing 0.15 mg Al/mL of $Al(OH)_3$ gel) and saline respectively 14 d after the last immunization of vaccine. Thickness of right and left hind paws was measured until 48 h after the challenge with Dd and the maximum difference between the thickness of right and left paws was taken as the swelling reaction.

Statistical analysis

Analysis of the parallel line assays was performed by the Finney method.[42] Significance and validity tests were made at a level of $P = 0.05$ and confidence intervals were expressed at 95% probability level unless otherwise stated. Comparison of groups was made by t test to calculate P value for null hypothesis.

The outlier was detected by calculating F value using s^2 for all batches in a group and s^2 for batches excluding the most deviated one from the analysis. If the F value was significant, the most deviated value was considered an outlier.

Ethical statements

All animal work was performed in accordance with the Guidelines for Care and Use of Laboratory Animals of the National Institutes of Infectious Diseases. The procedures were approved by the Institutional Committee on Animal Care and Use.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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●原 著

成人百日咳の特徴と予後
—臨床的診断例における検査による診断確定群と非確定群の比較—

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要旨：2009年2月から2013年2月までに，咳持続期間と症状から臨床的百日咳の診断基準に合致した症例33例（男性11例，女性22例，平均年齢42.0±16.5歳）を対象にLAMP法，百日咳毒素抗体検査等を施行し，百日咳と確定診断できた症例が14例，確定できなかった症例が19例であった．百日咳確定群は咳持続期間が非確定群に比して有意に長く，高熱がないことが特徴であり，炎症反応上昇を認めなかった．さらに確定群では吸入ステロイド使用例や感染後に咳喘息や喘息を発症した症例が多くみられた．

キーワード：成人百日咳，気道過敏性，マクロライド，LAMP法
Adult pertussis, Bronchial hyperresponsiveness, Macrolide, Loop-mediated isothermal amplification method

緒 言

百日咳は，グラム陰性桿菌である百日咳菌 *Bordetella pertussis* によって引き起こされる急性の気道感染症である．百日咳菌は，気道上皮細胞，主として線毛細胞に付着して百日咳毒素を産生し，その結果激しい咳を生じるとされている．典型的にはカタル期（1〜2週間），痙咳期（4〜8週間），回復期（1〜2週間）という経過をとるが，痙咳期における連続性の咳嗽（staccato）や，吸気時の笛声音（whooping）が特徴的である．元来乳幼児の疾患として知られていたが，最近では成人においても増加していることが報告[1]され，注目を集めている．成人の百日咳感染は，症状がワクチン未接種の乳幼児のように典型的ではなく，そのため診断に苦慮し，適切な治療がなされない場合が多くみられる[2]．成人の百日咳は乳幼児の感染源となっている場合があり，ワクチン未接種の乳児が百日咳に感染すると無呼吸や肺炎などを合併し重篤となる危険性が高い．よって成人百日咳を早期診断し，抗菌薬を早期に投与することは重要であると思われる．

成人百日咳の診断において，咳嗽に関するガイドライン第2版では，臨床的診断と検査による確定診断をフローチャートに示している[3]．

今回我々は，臨床的百日咳の診断基準に合致した症例のなかで，検査により百日咳と確定できた症例（以下確定群）と，検査で百日咳と確定できなかった症例（以下非確定群）に分けて，その臨床的特徴と治療，予後について比較検討したので報告する．

対象と方法

2009年2月から2013年2月までに，咳を主訴に国立病院機構福岡病院内科を受診し，咳嗽に関するガイドライン第2版で臨床的に百日咳と診断された症例，すなわち14日間以上続く咳のうち，「発作性の咳き込み」，「吸気性笛声」，「咳き込み後の嘔吐」のいずれか1つ以上伴った症例33例（男性11例，女性22例，平均年齢42.0±16.5歳）を対象とした．

対象症例には，百日咳確定のため，鼻咽頭ぬぐい液を検体としてloop-mediated isothermal amplification（LAMP）法および培養，百日咳毒素（pertussis toxin：PT）に対する抗体価（抗PT抗体価），凝集素価（山口株：流行株K抗原：1.3.6）の測定を行った．抗PT抗体価は酵素免疫測定法（enzyme-linked immunosorbent assay：ELISA法），凝集素価は細菌凝集反応により測定した．LAMP法はKamachi[4]らの方法により，国立感染症研究所で測定した．なお，単血清を用いた凝集素価法は診断精度が低いとされているため，本研究ではペア血

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表1 背景因子の比較

	確定群				非確定群				p
	症例数	中央値	範囲		症例数	中央値	範囲		
			最低値	最大値			最低値	最大値	
年齢	14	52	18	76	19	33	20	71	0.008
受診までの咳持続期間（月）	14	1.00	0.50	2.25	19	0.75	0.50	2.00	0.392
咳持続期間（月）	10	1.63	1.50	4.00	14	1.40	0.75	2.50	0.024
白血球（/mm^3）	14	5,340	3,300	8,770	19	5,570	3,630	12,220	0.362
好中球（%）	14	54.5	38.1	79.8	19	59.8	32.3	75.4	0.259
リンパ球（%）	14	32.9	16.4	46.5	19	26.2	16.0	56.4	0.251
好酸球（%）	14	3.1	0.8	31.1	19	3.3	0.4	28.7	0.702
CRP（mg/dl）	14	0.00	0.00	0.44	19	0.30	0.00	3.00	0.016

表2 性，年齢，喫煙，白血球数，CRP値と百日咳（確定例）との関係

	確定群	非確定群	調整オッズ比*	95%信頼区間		p値
				下限	上限	
性						
女性	8	14	1.00	基準群		
男性	6	5	1.55	0.15	16.33	0.716
年齢						
40歳以下	4	15	1.00	基準群		
41歳以上	10	4	60.10	2.21	1632.82	0.015
喫煙						
非喫煙	9	13	1.00	基準群		
現在あるいは過去喫煙	5	6	1.27	0.12	14.00	0.843
白血球数（/mm^3）						
5,000以下	5	3	1.00	基準群		
5,001～8,000	8	12	3.90	0.14	107.79	0.422
8,001以上	1	4	0.22	0.01	9.09	0.426
					傾向性	0.645
CRP値（mg/dl）						
0.3未満	12	9	1.00	基準群		
0.3以上	2	10	0.05	0.003	0.93	0.045

*他の変数を相互に調整.

清を用いた.

以下の基準を1つ以上満たす症例を確定群，満たさなかった例を非確定群とした．①LAMP法陽性，②培養陽性，③シングル血清で抗PT抗体価が100EU以上，④ペア血清で抗PT抗体価2倍以上変化，⑤ペア血清で山口株4倍以上変化.

さらに血中総IgE値，RAST値を測定し，スパイロメーターで，努力性肺活量，1秒量，V_{50}，V_{25}，最大吸気量などを測定した.

両群で，臨床症状，治療や予後についても比較検討した.

本研究は，事前に独立行政法人国立病院機構福岡病院の倫理委員会の承認を得たうえで，対象者の同意を得て行った.

統計処理：両群間の比較は，Mann-WhitneyのU検定を用いた．臨床症状，年齢，予後の比較はχ^2検定を用いたが，各カテゴリーの期待値の最小値が5未満である場合には，フィッシャーの直接確率検定（原則両側検定）を用いた．とくに断らない限り，$p<0.05$であるときを統計学的有意差ありとした．また，性，年齢，喫煙，および百日咳でしばしば増多する白血球数，通常は上昇しないCRP値と，百日咳確定診断との関係を，多重ロジスティック回帰分析の手法を用いて検討した．統計解析には，StatView-J 5.0とSTATA11.1を用いた.

結　果

33対象症例のうち，確定群は14例（男性6例，女性8例，年齢中央値52歳），非確定群19例（男性5例，女性14例，年齢中央値33歳）であった．確定群の内訳は，診断基準で①のみ満たすものが1例，②のみ0例，③の

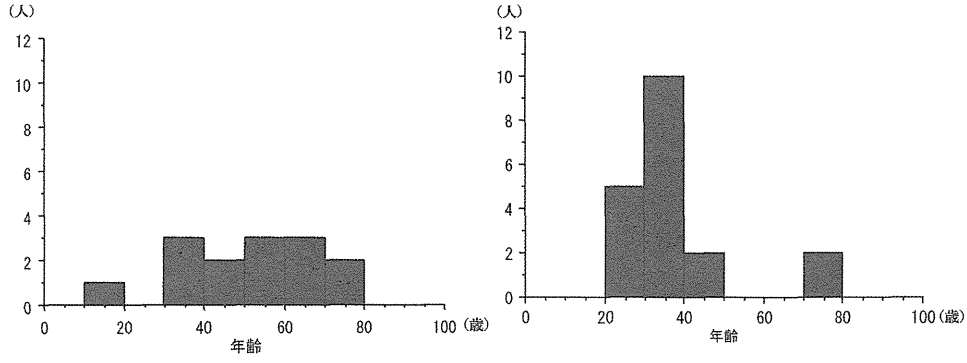

図1　確定群と非確定群の年齢分布．確定群に比して，非確定群は40～60代が少なく，40歳を超える症例は有意に確定群が多かった．

表3　臨床症状の比較

	確定群 (あり/なし)	非確定群 (あり/なし)	p
周囲の咳	5/8	12/6	0.119
発作性の咳き込み	11/3	17/2	0.628
吸気性笛声音	3/11	5/14	1.000
咳き込み後嘔吐	5/9	5/14	0.707
夜間覚醒	8/6	14/5	0.459
窒息しそうな咳	7/7	10/9	0.881
胸痛	6/8	6/13	0.506
喘鳴	3/11	2/17	0.628
37.5℃以上の発熱	0/14	5/14	0.057

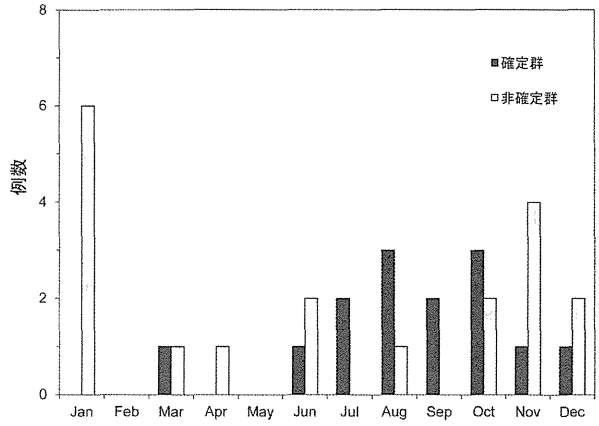

図2　確定群と非確定群の発症月別症例数．確定群では7～10月の夏～秋が多く，非確定群は1月に多く認められた．

み7例，④のみ3例，⑤のみ2例，①と②を満たすものが1例であった．

両群の背景因子を表1に示した．表2に，性，年齢，喫煙，白血球数，およびCRP値と，百日咳確定診断との関係をオッズ比で示した．年齢は確定群が有意に高く（p＝0.008），さらに年齢の分布をみると，非確定群では40～60代が少なく，40歳を超える症例は有意に確定群が多かった（図1，表2）．非確定群には女性（14例）が多く認められたが，性差での有意差を認めなかった．受診までの咳持続期間に有意差はなかったものの，発症から完治までの咳持続期間において確定群が有意に長かった（p＝0.024）．また，白血球やリンパ球数の比較では，両群間に有意差を認めなかった．炎症反応のCRPについては，非確定群が有意に高値を示し，確定群では上昇を認めなかった（p＝0.016）（表1）．

臨床症状の比較では，非確定群に37.5℃以上の発熱者5名が認められ，確定群では有意に発熱者が少ないことが示された［p＝0.057（両側検定），0.049（片側検定）］（表3）．なお，乳幼児に特徴的な症状（発作性の咳き込み，吸気性笛声音，咳き込み後の嘔吐）については両群に有意差を認めなかった．同様にスパイロメーターでの検査値［努力性肺活量（FVC），1秒量（%FEV_1），1秒率（FEV_1/FVC%），50%，25%肺活量でのV_{max}（%V_{50}，%V_{25}），最大吸気量（IC）］には両群間で有意差を認めず，IgEも両群間で有意差を認めなかった．特異的IgEはハウスダスト，ダニ，カンジダ，スギをCAP法で比較したところ，スギのRASTが確定群で有意に低値を示した（p＝0.038）（表4）．

図2に発症月別の症例数を示した．確定群では7～10月の夏～秋が多く，非確定群では1月に多く認められた．

確定群では14例中7例（50%），非確定群では19例中7例（37%）にマクロライド系抗菌薬が投与されていた．両群で，マクロライド系抗菌薬の投与例と非投与例で咳の持続期間に有意差を認めなかった．また確定群で発症から2週間以内のマクロライド系抗菌薬投与例と2週間以上経過したあとの投与例でも咳持続期間に有意差を認

表4 呼吸機能・IgEの比較

	確定群				非確定群				p
	症例数	中央値	範囲		症例数	中央値	範囲		
			最低値	最大値			最低値	最大値	
%FVC	12	101.4	85.4	124.7	14	102.0	61.4	111.3	0.396
$\%FEV_1$	12	102.8	73.5	113.5	14	94.6	54.9	110.0	0.165
$FEV_1/FVC\%$	12	81.9	63	89.3	14	83.8	62.9	98.6	0.589
$\%V_{50}$	12	79.7	29.7	133.6	14	70.5	24.4	91.9	0.122
$\%V_{25}$	12	65.8	18.3	112.6	14	66.9	8.8	110.4	0.758
IC (L)	12	2.32	1.34	3.62	14	2.11	1.36	3.05	0.589
IgE	13	136	10.9	8,517	14	53.6	10.3	1,021	0.225
RAST									
ハウスダスト	12	0	0	4	14	0	0	5	0.347
ダニ	12	0	0	4	14	0	0	5	0.347
カンジダ	11	0	0	3	13	0	0	0	0.116
スギ	11	0	0	4	14	1.5	0	5	0.038

表5 予 後

	確定群 (あり/なし)	非確定群 (あり/なし)	p
気道過敏性(メサコリン負荷)	4/2	1/1	1.000
最終的にICS使用	8/6	4/15	0.033
CVA, BAの発症	5/9	1/18	0.062

ICS：inhaled corticosteroid, CVA：cough variant asthma, BA：bronchial asthma.

めなかった.

予後については，確定群で6例にアストグラフ法による気道過敏性検査を施行し，うち4例に気道過敏性の亢進を認めた．非確定群では気道過敏性検査は2例のみ実施され，うち1例に過敏性亢進を認めた（表5）．さらに咳が長引くため，最終的治療として吸入ステロイドを投与された症例は確定群で8例，非確定群で4例であり，有意に確定群で多かった（p＝0.033）(表5)．また最終的に咳喘息や気管支喘息を発症した症例が確定群で5例(35.7%)，非確定群で1名（5.3%）であり，有意差は認めなかったが，確定群で多い傾向にあった（p＝0062)．

考 察

我が国では，1950年に百日せきワクチンが予防接種法に定められ，ワクチンの接種率増加とともに，百日咳感染症例は減少してきたが，2006年より罹患数が増え始め，2007年には集団感染も発生した[5]．増加のほとんどが成人であり，注目を集めている．成人の百日咳感染は，臨床症状だけで診断することは容易ではない．そのため，百日咳感染の診断が遅れ，ワクチン未接種の乳幼児への感染源となる可能性が指摘されている．成人百日咳を早期診断，早期治療することは，乳児の重症化を防ぐという意味において重要と考えられる．

2012年に発刊された日本呼吸器学会編集の咳嗽に関するガイドライン第2版では，百日咳の診断は臨床診断と検査による確定診断に分類され，フローチャートに示されている．今回我々は，このフローチャートに従い，臨床的に百日咳と診断した症例を，検査により診断が確定した群と確定できなかった群に分けて，その臨床的特徴や背景因子を比較するとともに，その治療・予後を検討した．なお非確定群において，百日咳感染を完全には否定できない症例も含まれている可能性はあるが，その後外来で管理し，PT抗体価の再検査を施行し上昇を認めておらず，大部分の症例は，百日咳感染が否定されうると考えている．

年代の比較では，確定群は各年代層にわたり，性別には差を認めなかった．一方非確定群では，確定群に比較して40歳を超える症例が有意に少なかった．その理由は明らかではないが，1970年代のワクチンによる副反応で一時的にワクチン接種率が低下したことなどと関係するかもしれない[6]．

両群で国立病院機構福岡病院受診までの期間には差を認めなかったが，確定群では発症からの咳持続期間が有意に長期であった．さらにワクチン未接種の乳幼児に特徴的に認められる白血球増多，特にリンパ球増多に関しても従来の報告と同様[7]に成人では認められず，非確定群との間に有意差を認めなかった．リンパ球を増加させるのは百日咳毒素（PT）とされている．ワクチン既接種の成人はある程度の抗PT抗体を保有しているため，相対的なリンパ球増多を示さない可能性が指摘される．

ワクチン未接種乳幼児の百日咳の特徴である発作性の咳き込み，吸気性笛声音，咳き込み後の嘔吐や夜間覚醒，窒息しそうな咳，胸痛，喘鳴などの症状発現は両群で有

意差を認めなかった．37.5℃以上の発熱例は，確定群にはみられなかった．De Serres ら[8]は，成人百日咳感染664例において，臨床症状を検討し，発作性咳嗽が全体の99%，無呼吸が87%，笛声音69%，咳嗽後の嘔吐が65%にみられたと報告している．今回の検討では，確定群で，発作性の咳き込み78.6%(11/14)，吸気性笛声21.4%(3/14)，咳き込み後嘔吐35.7%（5/14）であった．Miyashita ら[9]は，細菌学的検査により百日咳と診断された例と百日咳以外の例を比較検討して，発作性の咳き込み，咳き込み後の嘔吐，吸気性笛声音は百日咳群が有意に多く，周囲に咳をしている人がいた割合も高かったと報告している．しかし発作性の咳き込みは特異度が低く，逆に嘔吐や笛声音に関しては，感度は低いが特異度は高いとしている．さらに，呼気中の一酸化窒素濃度（FeNO）を測定し，結論として，咳き込み後の嘔吐や吐き気があり，FeNOが正常であれば百日咳感染も考慮しなくてはいけないとした（感度72%，特異度70%）．本研究では臨床的百日咳患者，すなわち2週間以上咳が続き，百日咳に特徴的とされる発作性の咳き込み，吸気性笛声音，咳き込み後嘔吐のいずれか1つの症状がある患者を対象としたことから，確定群と非確定群間で症状の有意差が出なかったと考察された．なお，百日咳の発症時期に関して，Miyashita らは5〜8月が多く，百日咳以外の症例は12〜2月が多いと報告しており，本研究でも同様な成績が得られた．

百日咳の診断基準については，2000年のWHOの基準[10]をはじめ，各国で定められてきた．我が国では2008年に診断基準案[11]が出され，2012年の呼吸器学会ガイドライン[3]では14日間以上続く咳に，発作性の咳き込み，吸気性笛声音，咳き込み後嘔吐があれば臨床的に診断可能とされ，咳の持続期間によって診断のフローチャートがまとめられている．

乳幼児へのワクチンの普及によって，相対的に成人での百日咳感染が増加し，臨床症状の多様性が認められてきた．2012年にGlobal Pertussis Initiative Roundtable Meetingでのサマリー[12]が報告され，年齢別の臨床症状がまとめられた．それによると0〜3ヶ月，4ヶ月〜9歳，10歳以上の3つの年齢層に分けて，臨床症状と所見を表したアルゴリズムを提唱し，10歳以上では発作性で痰を伴わない咳が1週間以上続き，発熱と膿性を伴わない鼻風邪様症状が臨床的な特徴であると報告されている．

Miyashita ら[5]は抗菌薬投与時期と咳嗽の持続期間について検討を行い，咳嗽発症から2週間以内に投与すると咳持続期間が有意に短縮されると報告している．そのため，咳嗽症状の改善には早期診断，早期治療が有用であると考察されるが，本研究では症例数が少なく抗菌薬(特にマクロライド系)投与と咳持続期間に有意差を認めることはできなかった．

百日咳の予後に関して，これまでに百日咳感染と気道過敏性との関係は明らかとなっていない．本研究では百日咳確定群に喘息や咳喘息の発症例が多く認められたことから，百日咳感染により気道上皮が障害され，その結果気道過敏性を獲得した可能性が指摘される．なお，マイコプラズマなどの非定型肺炎後に喘息を発症した報告[13]もあり，喘息発症の一つの要因として百日咳感染も考慮されるべきものである．今後，百日咳感染と気道過敏性との関係については詳細な検討が必要である．

臨床症状のみで百日咳感染を診断確定することは困難であり，臨床診断の正確性には限界があると思われる．実地臨床ではそれをふまえたうえで臨床診断基準を用いるべきと考える．すなわち，臨床的に百日咳と診断された例のなかにも，検査で確定できなかった，百日咳感染以外の症例が含まれている．臨床的に本症を疑った場合には抗体価測定やLAMP法での確認が重要と思われる．長引く咳を主訴とする症例において，臨床症状だけで百日咳を確定診断することは容易でないが，発熱がなく，炎症反応も乏しい場合は百日咳感染も鑑別に入れて治療していくことが必要である．

著者のCOI（conflicts of interest）開示：岡田　賢司；講演料（ファイザー，MSD）．他は本論文発表内容に関して特に申告なし．

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Abstract

Clinical features of pertussis in adults

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To evaluate the clinical features of pertussis infection in adults, we examined 33 subjects (11 males and 22 females; mean age, 42.0±16.5 years) who were clinically diagnosed to have pertussis infections according to guidelines of the Japanese Respiratory Society. All subjects were examined for medical history, family history, and respiratory function, and serum samples and nasopharyngeal swabs were collected. The nasopharyngeal samples were analyzed by loop-mediated isothermal amplification (LAMP). The serum specimens were assayed for antibodies to pertussis toxin (PT) by an enzyme-linked immunosorbent assay. Fourteen patients were diagnosed as having a pertussis infection based on the values of the PT and/or the nasopharyngeal swab (LAMP). The other 19 subjects were not diagnosed. The duration of coughing in pertussis subjects was significantly longer than in the other cough subjects. No pertussis subjects had a high fever (>37.5℃). Five subjects (35.7%) developed cough variant asthma or bronchial asthma postpertussis. In adult patients with persistent coughs, especially in those without high fevers, a pertussis infection should be considered as a differential diagnosis.

Global Population Structure and Evolution of *Bordetella pertussis* and Their Relationship with Vaccination

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ABSTRACT ***Bordetella pertussis*** **causes pertussis, a respiratory disease that is most severe for infants. Vaccination was introduced in the 1950s, and in recent years, a resurgence of disease was observed worldwide, with significant mortality in infants. Possible causes for this include the switch from whole-cell vaccines (WCVs) to less effective acellular vaccines (ACVs), waning immunity, and pathogen adaptation. Pathogen adaptation is suggested by antigenic divergence between vaccine strains and circulating strains and by the emergence of strains with increased pertussis toxin production. We applied comparative genomics to a worldwide collection of 343** ***B. pertussis*** **strains isolated between 1920 and 2010. The global phylogeny showed two deep branches; the largest of these contained 98% of all strains, and its expansion correlated temporally with the first descriptions of pertussis outbreaks in Europe in the 16th century. We found little evidence of recent geographical clustering of the strains within this lineage, suggesting rapid strain flow between countries. We observed that changes in genes encoding proteins implicated in protective immunity that are included in ACVs occurred after the introduction of WCVs but before the switch to ACVs. Furthermore, our analyses consistently suggested that virulence-associated genes and genes coding for surface-exposed proteins were involved in adaptation. However, many of the putative adaptive loci identified have a physiological role, and further studies of these loci may reveal less obvious ways in which** ***B. pertussis*** **and the host interact. This work provides insight into ways in which pathogens may adapt to vaccination and suggests ways to improve pertussis vaccines.**

IMPORTANCE **Whooping cough is mainly caused by** ***Bordetella pertussis*****, and current vaccines are targeted against this organism. Recently, there have been increasing outbreaks of whooping cough, even where vaccine coverage is high. Analysis of the genomes of 343** ***B. pertussis*** **isolates from around the world over the last 100 years suggests that the organism has emerged within the last 500 years, consistent with historical records. We show that global transmission of new strains is very rapid and that the worldwide population of** ***B. pertussis*** **is evolving in response to vaccine introduction, potentially enabling vaccine escape.**

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Bordetella pertussis is the primary causative agent of pertussis (whooping cough), a respiratory disease which is particularly severe for unvaccinated infants. Indeed, pertussis was a major cause of infant deaths before the introduction of vaccination. Even today, pertussis is a significant cause of child mortality, and estimates from the WHO suggest that, in 2008, about 16 million cases of pertussis occurred worldwide, 95% of which were in developing countries, and that about 195,000 children died from this disease (1).

There has been much speculation about the origin of pertussis. Although the disease has very characteristic symptoms and high mortality in unvaccinated children, references to pertussislike symptoms have not been found in the ancient European literature. The first documented pertussis epidemic occurred in Paris in 1578 (2). In the 16th and 17th centuries, descriptions of pertussis epidemics in Europe were documented more frequently in the literature, possibly suggesting an expansion of the disease (3). The apparent emergence of pertussis in Europe over the last 600 years may be due to import, as symptoms similar to pertussis were described in a classical Korean medical textbook from the 15th century (4).

The introduction of vaccination has significantly reduced the pertussis burden; however, in the 1990s, a resurgence of pertussis was observed in many highly vaccinated populations (5). The years 2010 to 2012 have seen particularly large outbreaks in Australia, the Netherlands, the United Kingdom, and the United States, with significant mortality in infants (6–10). The possible causes for the pertussis resurgence are still under debate and include waning vaccine-induced immunity, the switch from whole-cell vaccines (WCVs) to less effective acellular vaccines (ACVs), and pathogen adaptation (5, 11–13). The contributions of these causes probably differ from country to country. The importance of pathogen adaptation is suggested by the antigenic divergence of circulating strains from vaccine strains and the emergence of strains which produce more toxin (reviewed in reference 5). Antigenic divergence initially involved relatively few mutations, affecting up to 12 amino acids in the five *B. pertussis* proteins included in ACVs, i.e., filamentous hemagglutinin (FHA), pertactin (Prn), the Ptx A subunit (PtxA), serotype 2 fimbriae (Fim2), and serotype 3 fimbriae (Fim3). In the 1980s, strains emerged with a novel allele for the Ptx promoter, designated *ptxP3*. Strains carrying the *ptxP3* allele have been shown to produce more Ptx *in vitro* (14). Significantly, mutations in these six loci have been associated with clonal sweeps (15). The emergence of the *ptxP3* lineage is particularly remarkable because *ptxP3* strains have risen to predominance, replacing the resident *ptxP1* strains in many European countries, the United States, and Australia (14, 16–21). Furthermore, the emergence of *ptxP3* strains is associated with increases in pertussis notifications in at least two countries (14, 20). However, another study found that the resurgence of pertussis in the United States was correlated with the *fim3-2* allele and not with *ptxP3* (22). More recently, strains have emerged that do not express one or more components of pertussis vaccines, in particular, Prn and FHA (17, 23–25).

Together with at least 425 other genes, the genes for the five *B. pertussis* proteins used in ACVs belong to the so-called *Bordetella* virulence gene (Bvg) regulon, consisting of a sensory transduction system which translates environmental cues into changes in gene expression (26, 27). Low temperatures and high sulfate and nicotinic acid concentrations are signals known to suppress genes in the Bvg regulon (28). As essentially all known virulence-associated proteins require Bvg for their expression, Bvg activation is used to identify genes that play a role in the interaction with the host, even if the function of that gene is not known.

Key questions concerning pertussis are the origin of the disease, the forces that have driven the shifts in *B. pertussis* populations, and the role of these shifts in the resurgence of pertussis. To address these questions, we have determined the global population structure of *B. pertussis* by whole-genome sequencing of 343 strains from 19 countries isolated between 1920 and 2010. Phylogenetic analysis revealed a deep divergence between two lineages of *B. pertussis*, possibly suggesting two independent introductions of the organism from an unknown reservoir. Bayesian methods showed that the date of the common ancestor of one of these lineages correlates with the first descriptions of pertussis in Europe and that this lineage has increased in diversity subsequent to the introduction of vaccination. Our analyses revealed that many (putative) adaptive mutations occurred in the period in which the WCV was used, suggesting that vaccination was the major force driving changes in *B. pertussis* populations. Furthermore, we extend our previous observation that the mutation leading to the *ptxP3* allele occurred once and that the *ptxP3* strains have spread and diversified worldwide (29). Finally, we identified novel putative adaptive loci, the analysis of which may cast new light on the persistence and resurgence of pertussis and point to ways to increase the effectiveness of vaccination.

RESULTS AND DISCUSSION

Phylogeny and phylogeography of *B. pertussis*. We explored the evolutionary relationships among 343 *B. pertussis* strains collected from 19 countries representing six continents. Strains were isolated between 1920 and 2010 (Table 1; see Table S1 in the supplemental material). Illumina reads were aligned to the reference genome *B. pertussis* Tohama I (30), and 5,414 single-nucleotide polymorphisms (SNPs) were identified (Table S2), corresponding to a mean SNP density of 0.0013 SNPs/bp and an estimated mutation rate of 2.24×10^{-7} per site per year. We generated a maximum likelihood phylogeny representing the *B. pertussis* global population structure (Fig. 1A; Fig. S1). This phylogeny revealed two deep branches separated by 1,711 SNPs. Branch I contained only a small number of strains (1.7%), which were isolated between 1954 and 2000 and harbor *ptxA5* and *ptxP4* alleles (coding for the Ptx A subunit and the Ptx promoter, respectively), which are infrequently isolated nowadays. This branch includes the type strain 18323. Branch II contained strains isolated between 1920 and 2010 which fall into the more common *ptxA2 ptxP1*, *ptxA1 ptxP1*, and *ptxA1 ptxP3* types (Fig. 1B). Bayesian phylogenetic analysis estimated that these two lineages diverged around 2,000 years ago (median, 2,296 years; 95% confidence interval [CI], 1,428 to 3,340), which may reflect the loss of intermediate lineages over time or may represent two independent introductions of *B. pertussis* into the global human population from an unknown reservoir. The adaptation of *B. pertussis* to the human population has been postulated to have involved a significant evolutionary bottleneck and was associated with considerable gene loss and gene inactivation due to insertion sequence (IS) element expansion and mutations (30), a process commonly seen in host-restricted bacteria (31). In the analysis of the Tohama I genome sequence, it was estimated that up to 25% of genes were lost relative to those present in the common ancestor with *Bordetella para-*

TABLE 1 Geographic origin and period of isolation of *B. pertussis* strains used in this study

Continent	Country	No. of strains	Isolation period	Introduction of vaccination
Africa	Kenya	17	1975	1980s
	Senegal	4	1990-1993	1980s
Asia	China	2	1957	Early 1960s
	Hong Kong	5	2002-2006	1950s
	Japan	17	1988-2007	1950s
	Taiwan	23	1992-2007	1954
Australia	Australia	37	1974-2007	1953
Europe	Denmark	9	1962-2007	1961
	Finland	16	1953-2006	1952
	France	11	1993-2007	1959
	Italy	15	1994-1995	1995
	Netherlands	60	1949-2010	1953
	Poland	16	1963-2000	1960
	Russia	2	2001-2002	1956-1959
	Sweden	23	1956-2006	1953
	United Kingdom	20	1920-2008	1957
North America	Canada	17	1994-2005	1943
	USA	36	1935-2005	1940s
South America	Argentina	13	1969-2008	1970s
Total		343	1920-2010	

pertussis (30), and 9.5% of those remaining were inactivated and were only present as pseudogenes. A manual comparison of 50% of the pseudogenes in Tohama I and strain 18323 (representing the two deep branches) showed that 72% of the pseudogenes were shared, and of those, all had identical inactivating mutations (Table S3). This indicates that the host restriction of *B. pertussis* and the associated bottleneck occurred before the divergence of these two lineages and long before the first description of the disease. The most parsimonious explanation would suggest that this process involved adaptation to the human host, and this would indicate that pertussis was introduced into the global population twice from a reservoir in an unsampled human population or that the intermediate diversity has been lost. The alternative explanation, that the adaption was to another host, would require both an unknown reservoir species and two separate introductions into the human population.

Three vaccine strains were included in this study, Tohama I and two American strains (strains B308 and B310) (see Table S1 in the supplemental material), which were placed in branch II. The vaccine strains and the reference genome, Tohama I, both represent lineages and antigenic genotypes for which recent isolations are rare. Most recent *B. pertussis* isolates stem from a lineage (lineage IIb) within branch II, which appeared before the introduction of vaccination but has expanded since. A Bayesian phylogenetic and skyline analysis of isolates from lineage IIb for which isolation date information was available (Fig. 1B; Fig. S2) reveals that there was no evidence of loss of diversity (represented by the effective population size) after the introduction of vaccination. This was unexpected, as one would assume that the introduction of vaccination would lead to a decrease in population diversity, as the selective pressure may lead to a population bottleneck whereby only those lineages that escape the vaccine may survive. Indeed, some previous studies have observed such a decrease in population diversity following the introduction of vaccination. However, these studies were based on geographically more restricted pathogen populations (32–34). Our results suggest that, despite whole-cell vaccines reducing the prevalence of many of the older lineages, they have not been eradicated completely, so the diversity of these lineages is still present in the *B. pertussis* population. The explanation for this may be that such lineages have persisted in geographical regions where vaccination has not become routine. In fact, there is some evidence from the skyline analysis that population diversity increased in lineage IIb after vaccine introduction. Although the effect of sampling density before and after vaccine introduction is unclear, the shape of the phylogenetic tree suggests that this increase was primarily the result of the expansion of the *ptxA1* lineage, which may represent some level of vaccine escape in countries where vaccination had been introduced.

A second increase in effective population size (diversity) coincides with the emergence and expansion of a lineage carrying the *ptxP3* allele. In the mid-1990s, there appears to be a drop in diversity, correlating with the loss of a number of early *ptxA1* lineages, and perhaps corresponding with the introduction of the ACV in the mid-1990s. However, diversity very quickly increased again with the expansion of a sublineage of the *ptxP3* group that acquired a *fim3-2* allele, again suggesting the selection and diversification of vaccine escape lineages.

There is little evidence of geographical structure in the phylogenetic tree (Fig. 1B). The sampling of the older branch I lineages is sparse in space and time, making inference difficult. However, the *ptxA1* lineage is clearly dispersed globally, and the *ptxP3* and *fim3-2* lineages show no geographical clustering at all, indicating that there has been very rapid global spread of these recently evolved lineages.

Temporal trends in frequencies of alleles coding for vaccine components. To explore the influence of vaccination on the *B. pertussis* population, we focused on genes coding for antigens known to induce protection and included in modern ACVs, including serotype 2 fimbriae (*fim2*), serotype 3 fimbriae (*fim3*), pertactin (*prn*), and the A subunit of Ptx (*ptxA*) (5, 35). Although it is used in ACVs, filamentous hemagglutinin was not included, as accurate assembly and assignment of SNPs was not possible due to the presence of repeats and paralogs. As previous studies suggest that variation in the Ptx promoter, *ptxP*, was linked to clonal sweeps (15, 21, 33), we also included *ptxP* alleles in our analyses.

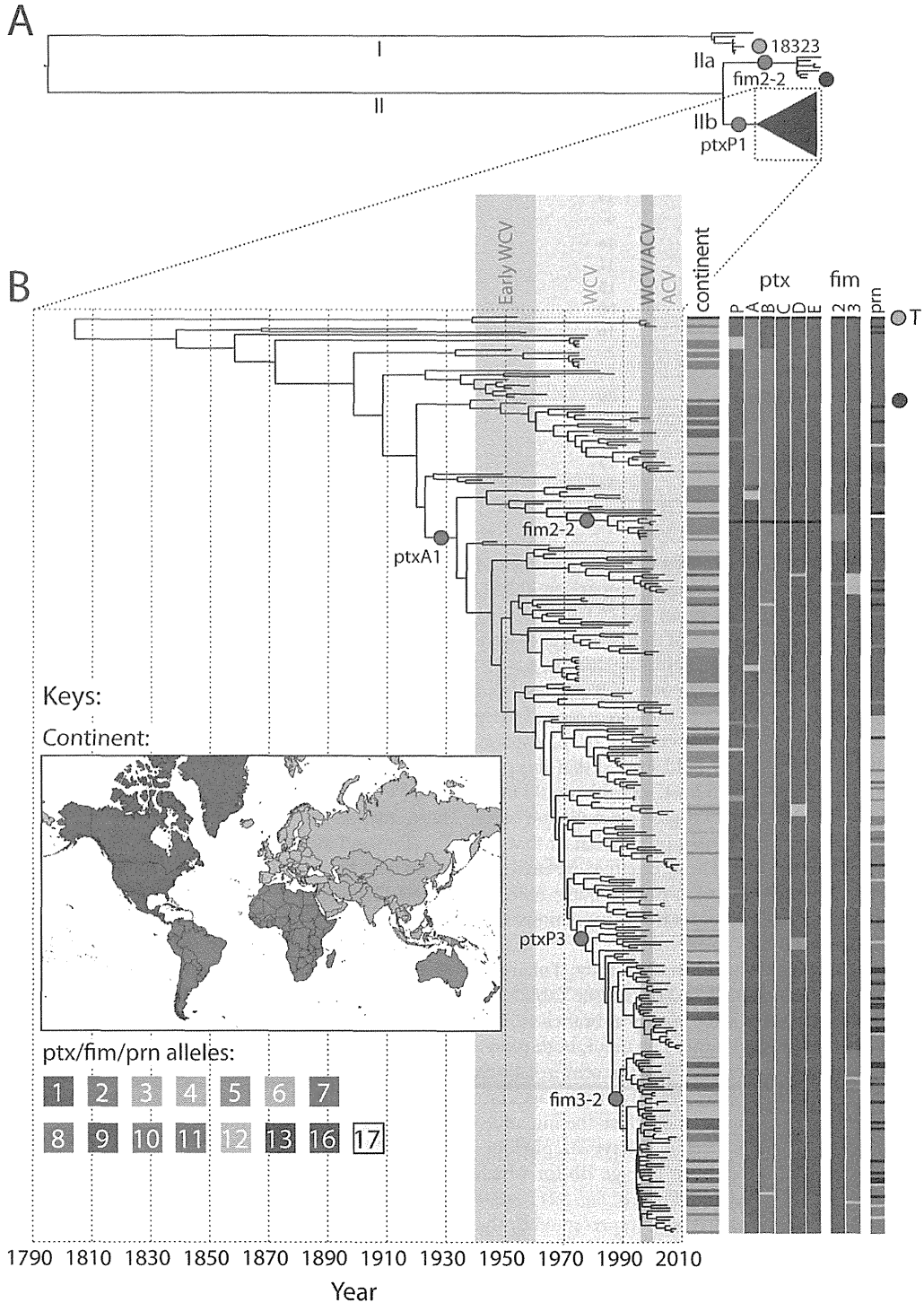

FIG 1 Global phylogeny of *B. pertussis*. (A) Outline of the maximum likelihood phylogeny of all *B. pertussis* samples sequenced, showing the deep divergence between lineages I and II. The complete tree is shown in Fig. S1 in the supplemental material. (B) Bayesian phylogeny of samples for which date information was available within the most common clade of *B. pertussis*. The position of a node along the *x* axis of the tree represents the median date reconstructed for that node across all sampled trees. Dates of whole-cell vaccine (WCV) and acellular vaccine (ACV) periods are shown as background colors behind the tree. To the right of the tree, the continent of origin of isolates is indicated by the first column of horizontal bars, colored according to the inset key. The remaining nine columns represent loci within the *ptx* operon, the *fim2* and *fim3* loci, and the *prn* locus, with assigned numerical alleles colored according to the key. The positions of reference strains 18323 and Tohama I (T) are indicated in panels A and B with green filled circles. Black filled circles represent the American vaccine strains B308 (A) and B310 (B) (Table S1). Red circles indicate the major changes in antigen gene alleles in proteins used in current ACVs (from *ptxA2* to *ptxA1*, *fim2-1* to *fim2-2*, *ptxP1* to *ptxP3*, and *fim3-1* to *fim3-2*).

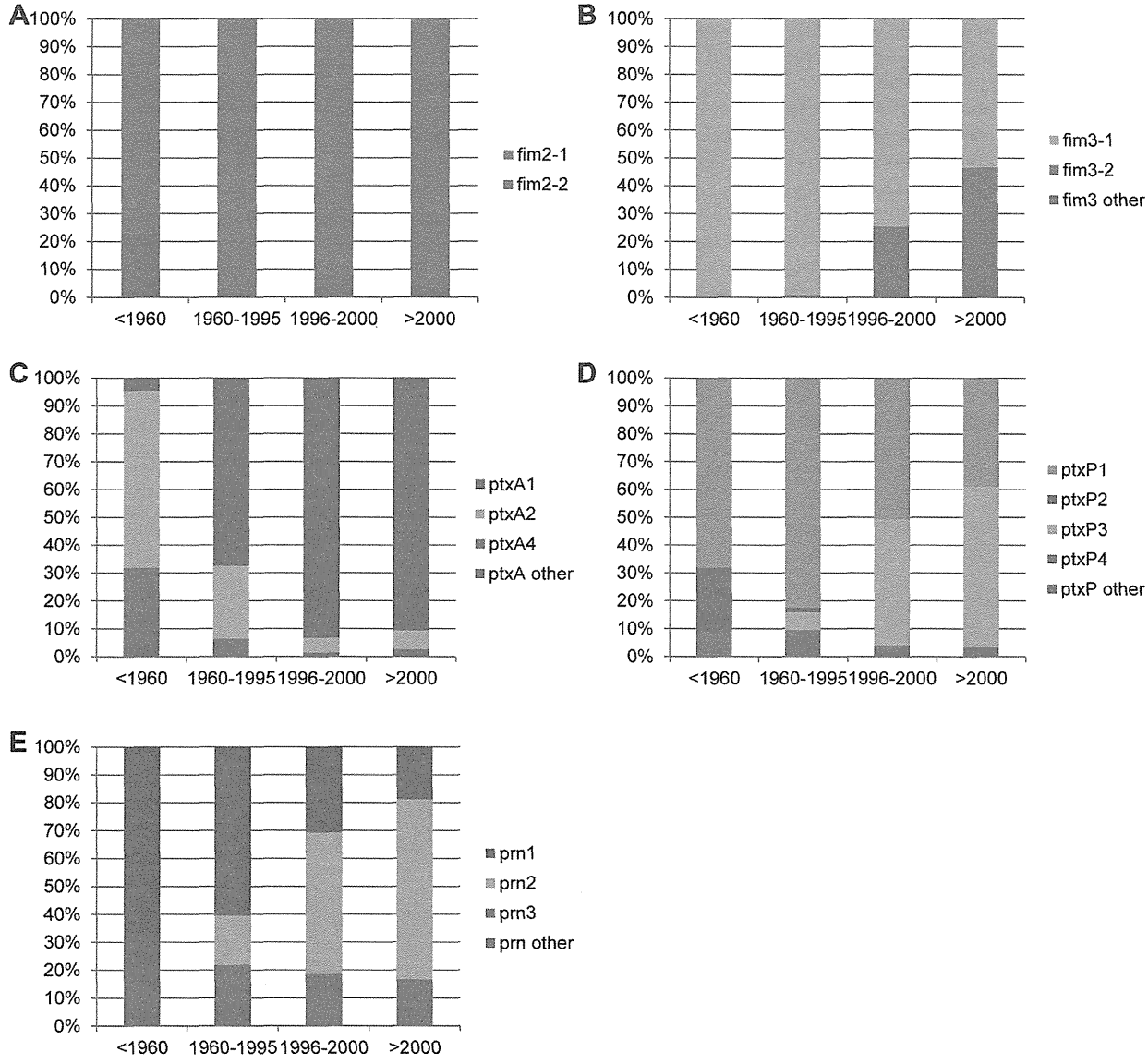

FIG 2 Temporal trends in strain frequencies for the *fim2* (A), *fim3* (B), *ptxA* (C), *ptxP* (D), and *prn* (E) alleles. Four periods were defined to reflect the worldwide changes in pertussis vaccination, the early WCV period (earlier than 1960), the period in which mainly WCVs were used (WCV period, 1960 to 1995), the period in which both WCVs and ACVs were used (WCV/ACV period, 1996 to 2000), and a period in which mainly ACVs were used (ACV period, later than 2000).

With the exception of *ptxA10*, *prn16*, and *prn17*, all alleles have been described before, and references and accession numbers are given in Text S1 in the supplemental material. The major changes in antigen gene alleles (from *ptxA2* to *ptxA1*, *fim2-1* to *fim2-2*, *ptxP1* to *ptxP3*, and *fim3-1* to *fim3-2*) are marked on the nodes in the phylogenetic tree in Fig. 1B. In most countries, vaccination was introduced between 1940 and 1960 (Table 1), and worldwide, many different *B. pertussis* strains have been used to produce vaccines. A compilation of 23 vaccine strains revealed that the most prevalent alleles found in vaccine strains were *fim2-1* (82%), *fim3-1* (100%), and *prn1* (74%) or *prn7* (22%) (Table S4). If one Dutch, one Swedish, and one acellular vaccine strain were omitted, all other vaccine strains carried the *fim2-1*, *fim3-1*, and *prn1/7* alleles. More diversity in vaccine strains was observed with respect to *ptxA*, for which four alleles, *ptxA1*, *ptxA2*, *ptxA3*, and *ptxA4*, were observed at frequencies of 13%, 52%, 4%, and 31%, respectively. For twelve vaccine strains, the *ptxP* allele has been determined. The *ptxP1* allele and *ptxP2* allele were found in 67% and 33%, respectively. Most ACVs are derived from two strains, Tohama I and 10536, which carry the alleles *fim2-1*, *fim3-1*, *prn1*, *ptxA2*, and *ptxP1* and *fim2-1*, *fim3-1*, *prn7*, *ptxA4*, and *ptxP2*, respectively.

To investigate temporal trends in allele frequencies, we defined four periods to reflect the worldwide changes in pertussis vaccination (Fig. 2): the early WCV period (earlier than 1960; $n = 22$), the period in which mainly WCVs were used (WCV period, 1960 to 1995; $n = 126$), the period in which both WCVs and ACVs were used (WCV/ACV period, 1996 to 2000; $n = 75$), and finally, a period in which mainly ACVs were used (ACV period, later than