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Alterations in the expression of *Bordetella pertussis* antigens in relation to the use of acellular pertussis vaccine in Finland

Vili Niinikoski [a], Alex-Mikael Barkoff [a,b], Jussi Mertsola [a,b], Jana Holubova [c], Jiri Masin [c], Peter Sebo [c], Qiushui He [a,b,*]

[a] Finnish Reference Laboratory for Pertussis and Diphtheria, Institute of Biomedicine, University of Turku, Turku, Finland
[b] InFLAMES flagship center, University of Turku, Finland
[c] Laboratory of Molecular Biology of Bacterial Pathogens, Institute of Microbiology of the Czech Academy of Sciences, Prague, Czechia

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ABSTRACT

Background: Bordetella pertussis isolates which do not express some of acellular pertussis vaccine (aPv) antigens, e.g. pertactin (PRN), have been increasingly reported in countries using aPvs. In Finland, primary pertussis vaccination with whole-cell vaccine was replaced by aPv containing pertussis toxin (PT) and filamentous hemagglutinin (FHA) in 2005 and then by aPv containing PT, FHA, and PRN in 2009. We aimed to study alterations in the expression of FHA, PRN, and PT, three antigens included in aPvs and adenylate cyclase toxin (ACT) not included in current aPvs, among Finnish isolates collected during 1991–2020.

Methods: Of 904 isolates collected by the Finnish Reference Laboratory for Pertussis during 1991–2020, 302 were randomly included. An adapted, monoclonal antibody based, antigen expression ELISA, including the culture of *B. pertussis* in Stainer-Scholte medium, was performed to quantify the expression of ACT, FHA, PRN, and PT of each isolate. ACT activity was also measured for 16 isolates. Arbitrary units were used for comparing levels of each antigen expression of isolates grouped in every five years.

Findings: Following the implementation of aPv in 2005, *B. pertussis* isolates exhibited a 1.75-fold increase for FHA ($p < 0.001$) and a 1.5-fold increase for ACT ($p < 0.0041$) expression until 2020. No FHA or ACT deficient isolates were detected. As the number of PRN deficient isolates has significantly increased with the time, the amount of PRN produced by the positive isolates has also started to decrease, especially after the use of aPv containing PRN. During this period, fluctuations in PT expression were observed.

Interpretation: The study demonstrated that in response to aPv-induced selection pressure, different types of selection of *B. pertussis* has occurred. For FHA and ACT, a steady increase in their production is observed, whereas the frequency of PRN deficient isolates is increased with time.

1. Introduction

Pertussis, also known as whooping cough, is a highly contagious respiratory infectious disease caused by the Gram-negative bacterium *Bordetella pertussis*. Notably in the aPv era, the waning of vaccine-induced immunity and evolution of the pathogen, such as alteration of expression of antigens are associated with the resurgence of pertussis [1]. In Finland, the whole cell vaccine has been used since 1952 and it was replaced for primary vaccination by aPvs in 2005. In Finland, the first Tdap3-booster vaccine (Boostrix; GlaxoSmithKline, Belgium) containing detoxified pertussis toxin (PT), filamentous hemagglutinin (FHA), and pertactin (PRN) was introduced to children aged 6 years in 2003 following the introduction of 2-component DTaP2-IPV-Hib-vaccine (Pentavac; Sanofi Pasteur MSD, France) containing PT and FHA was introduced for primary vaccinations in infants in 2005. General 2-component vaccine was replaced with 3-component DTaP3-IPV-Hib vaccine (Infanrix, GSK) in 2009. In 2019 Infanrix 3-component vaccine was again replaced with Pentavac 2-component vaccine. After 2009 Tdap3-boosters for military (2012) and adult 18–25 age (2018) has been added (Table 1) [2]. Since the last epidemic in Finland (2003–2004) and

Abbreviations: ACT, adenylate cyclase toxin;; aPv, acellular pertussis vaccine; FHA, filamentous hemagglutinin; PRN, pertactin; PT, pertussis toxin; wPv, whole-cell pertussis vaccine.

* Corresponding author at: Instutute of Biomedicine, University of Turku, Kiinamyllynkatu 10, 20520 Turku, Finland
E-mail address: Qiushui.He@utu.fi (Q. He).

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the introduction of aP vaccines, the vaccination coverage has been high and the incidence of pertussis has remained stable, with no signs of resurgence during 2005–2020) [2]. In addition, according to previous Finnish studies, no other defects in *B. pertussis* antigen production than PRN deficiency have been reported [3,4].

Alterations in multiple antigen expression of *B. pertussis* has been reported previously. These alterations include non-expression of acellular vaccine antigens PRN, PT, FHA, and fimbriae (FIM), but no deficiencies in expression of non-vaccine component adenylate cyclase toxin (ACT) has been found [4 – 7]. After introduction of aPvs, the first PRN and PT deficient isolates were reported in France in 2007 [8]. Later, isolates with deficiency mostly in PRN, but also in PT, FHA, and FIM deficiencies have been reported [9]. However, isolates comprising FHA and PT deficiency have remained low [4,10]. In addition, isolates with deficiency of two antigens, PRN-/FHA- and PRN-/PT-, have been reported [11,12], although they are rare. The association between time since aPvs were introduced and increase of PRN deficient isolates has been established [4].

In addition to antigen deficiencies, point mutation in promoter region of the gene encoding PT (*ptxP*) and alterations in FIM serotype, BrkA, and Vag8 has been reported [1], [13–15]. The isolates with the mutation in PT promoter region (*ptxP3*) emerged as early as 1990s, and after that has become dominant in many countries using aPvs [1]. In Finland, the first isolates with *ptxP3* mutation were detected in 1994 and became predominant since 2003 [16]. The mutation in *ptxP* is associated with increased expression of PT, which seemed to be associated with severe pertussis disease [1].

This study aimed to investigate the possible alterations in the expression level of *B. pertussis* antigens included (PT, FHA, PRN, and FIM2/3) and not included (ACT) in the aPvs using a newly developed assay. Further, the possible correlation between antigen alterations and introduction of aPv in Finland is examined with a large number of isolates collected during the period of 1991–2020.

2. Material and methods

2.1. Bacterial strains and cultures

Within the study period from 1991 to 2020, 904 clinical isolates of *B. pertussis* were collected in Finnish Reference Laboratory for Diphtheria and Pertussis, University of Turku, Finland. To attain comprehensive study set, 302 clinical isolates were randomly selected, considering the years with low isolate numbers of which all the isolates were included. Isolates were selected demographically to provide a comprehensive overview of circulating isolates in Finland. Among the selected isolates, 43 % were collected from Southwest Finland, 32 % from the Helsinki Metropolitan Area, and 25 % from other regions outside of these two areas. Isolates were isolated from patients from median age of 7.4 years ranging from under 1 month to over 70 years. Out of 302 selected isolates all expressed ACT, FHA, PT, and PRN except 47 which were PRN-deficient. Over 99 % of the isolates carried *ptxA1* and majority carried *prn2* and *ptxP3* alleles (Table 2). FIM serotype distribution fluctuated within the study period (Table 2). The isolates were grouped on 5-year time periods: 35 in 1991–1995, 60 in 1996–2000, 61 in 2001–2005, 34 in 2006–2010, 35 in 2011–2015, and 77 in 2016–2020.

Isolates were cultured on Bordet-Gengou medium containing 15 % sheep blood in 35 °C for 72 h. Grown colonies were re-cultured in 150 µl Stainer-Scholte medium on Microlon® high-binding microtiter plates (Greiner Bio-One GmbH, Frickenhausen, Germany) in shaker (+35 °C, 150 rpm) for another 24 h. The isolates were inoculated to Stainer-Scholte medium to reach bacterial density of 0.05 +/− 0.01. After 24 h culture, bacterial density reached 0.1 to 0.25. The bacterial densities were measured at 580 nm with plate reader (Victor Nivo™ multimode, Perkin Elmer, Turku, Finland). The *B. pertussis* strain Tohama I obtained from the culture collection of Institute Pasteur in Paris under the reference CIP 81.32 was used as a reference strain [17,18].

2.1.1. Quantitative ELISA-based assay protocol for measuring antigen expression levels of B. pertussis

The expression levels of FHA, PRN, PT, and ACT were detected using a specific indirect ELISA, as described in Fig. 1. The assay was conducted on Microlon® high-binding standard ELISA microtiter plates (Greiner Bio-One GmbH, Frickenhausen, Germany). Following the 24 h liquid culture in duplicates, bacterial growth was measured, and culture solutions were discarded. The wells were then washed three times with 0.9 % NaCl and 0.05 % Tween20 (Sigma, St. Louis, USA) washing buffer. Subsequently, antigen-specific monoclonal antibodies were added at a 1:1000 dilution in 2 % skimmed milk (100 µl/well) and incubated for 2 h. Following this, the wells were washed, and secondary antibodies were added in the same dilution as the monoclonal antibodies (100 µl/well). After a 2-h incubation, the wells were washed, and substrate (S0942-50TAB, Sigma, Helsinki, Finland) diluted in diethanolamine-MgCl buffer (Reagena, Toivala, Finland) was added (100 µl/well) and incubated for 10 min in the dark at room temperature. The reaction was stopped with a 3 M NaOH solution (3 M NaOH, Merck, Espoo, Finland) (100 µl/well) and the absorbance of the wells was measured at 405 nm using a plate reader (Perkin Elmer, Turku, Finland). The signal of

Table 1
Pertussis vaccination schedules and vaccines used in finland modified from [2].

YEAR	SCHEME	VACCINE COMPOSITION	CHANGE	VACCINE NAME (COMPANY)	STUDY COHORT	VACCINATION COVERAGE
BEFORE	–			No vaccine available	Older adults	
1952	–	DTwP	First only DwP, T added in 1957. 1962 change to DTwP (Fim2,3 strain), Additional Fim2 strain added in 1976	Diphteria-Pertussis Forte (Orion), PDT (Orion), DTwP (KTL)	Older and young adults	92–99 %
2003	3,4,5,20-24 m, 6y	DTwP + IPV + Hib Tdap3	- First aP booster vaccine in NIP	DTwP (KTL) Boostrix (GSK)	Adolescents Adults	98 %
2005	3,5,12 m 4y 14-15y	DTab3 – IPV – Hib DTab2 – IPV Tdap3	Introduction aP priming, 2 Bp component Also catch-up for 11-13y	Pentavac (Sanofi) Tetravac (Sanofi) Boostrix (GSK)	Adolescents and children Young adults	97–99 %
2009	3,5,12 m 4y 14-15y	DTaP3 – IPV – Hib DTaP2 – IPV Tdap3	3 Bp components - -	Infanrix (GSK) Tetravac (Sanofi) Boostrix (GSK)	Children Adolescents Young adults	99 %
2012	Military	Tdap3	Addition of pertussis vaccination in the military, 18-25y (estimation)	Boostrix (GSK)	Young adults	89–99 %
2018	25y	Tdap3	Introduction of adult booster	Boostrix (GSK)	Young adults	91 %
2019 (SEP)	3,5,12 m	DTaP2 – IPV – Hib	2 Bp components	Pentavac (Sanofi)	–	–

Table 2
Genotype and phenotype information on 302 selected *Bordetella pertussis* isolates in Finland, 1991–2020*

	1991–1995	1996–2000	2001-2005	2006–2010	2011–2015	2016–2020
Number of isolates	35	60	61	34	35	77
% of PRN–	–	–	–	–	20 % (7/35)	52 % (40/77)
% of *prn2*	71 % (25/35)	97 % (58/60)	95 % (58/61)	97 % (33/34)	89 % (31/35)	92 % (71/77)
% of *ptxA1*	100 % (35/35)	100 % (60/60)	100 % (61/61)	100 % (34/34)	94 % (33/35)	100 % (77/77)
FIM2/FIM3	33/0**	51/8**	19/39**	4/30	22/13	47/29*
ptxP1/ptxP3	25/0**	34/7**	21/40	3/30**	3/29**	0/76**

* Proportion of pertactin (PRN) deficient, *prn2* and *ptxA1* and number of isolates with FIM2/FIM3 and *ptxP1/ptxP3* alleles. In Finland, PRN deficient isolates were first detected in 2011.
** Data of majority of isolates were available.

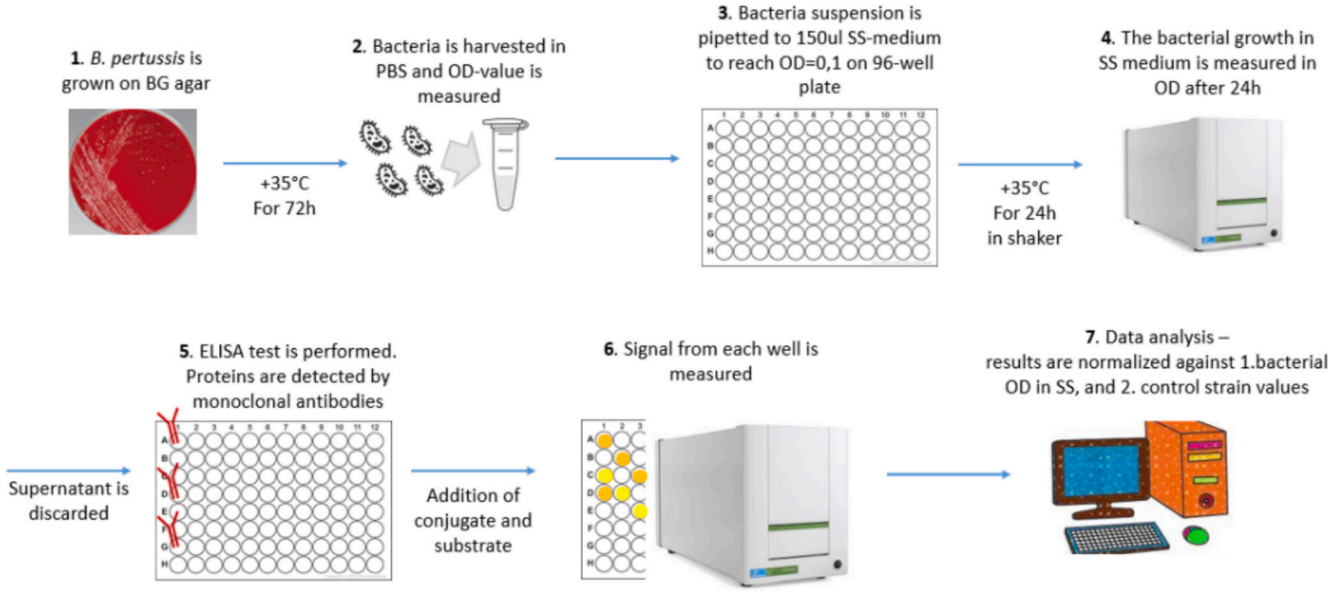

Fig. 1. Antigen expression assay workflow.

antigen-deficient isolates remained below 0.25 arbitrary unit (AU). To accommodate variations in bacterial growth and maintain measurement accuracy, the data obtained from the plate reader was normalized using both the bacterial growth (OD) and the measured expression value of the reference strain. Purified antigens of PT, FHA, PRN, and ACT were used as controls.

2.2. Validation of the antigen expression measurement method

2.2.1. Assay specificity

The assay specificity was assessed by comparing the antigen expression backgrounds of each *B. pertussis* isolate utilized previously with the results obtained from the current test (data not shown). The primary objective was to determine whether our assay yielded consistent results with previous tests regarding the expression or absence of expression of antigens by the isolates. The expression background of the isolates was previously studied with assay introduced by Barkoff et al. [3,5].

2.2.2. Assay variability

To show that our ELISA-based expression assay was consistent and precise, inter- and intra-assay variation tests were performed. Intra-assay tests were performed by using five independent wells/replicates containing the same isolate and performed for each antigen. Inter-assay tests were performed using three different *B. pertussis* isolates in three independent expression tests for each antigen. In each individual expression test triplicate wells per antigen was used. Coefficient of variation (CV) was used to demonstrate the precision of the assay.

For inter-assay variation, CV of 15 % and for intra-assay variation CV of 10 % was considered as an upper limit of acceptance.

2.2.3. Assay accuracy

Linear ranges were interpreted by using 10 different dilutions (0.165 µg, 0.33 µg, 0.66 µg, 1.0 µg, 2.0 µg, 3..µg, 5.0 µg, 6.66 µg, 8.0 µg, and 10.0 µg) of each purified antigen. The purified FHA, PRN, and PT were kindly provided by GSK, Belgium, and the purified ACT by Peter Sebo, Institute of Microbiology of the Czech Academy of Sciences, Prague, Czech Republic. The absorbance of each antigen was measured with plate reader (Perkin Elmer, Turku, Finland) with 405 nm wavelength.

2.3. Determination of AC activity

To detect AC activity and explore potential correlations with ACT expression, 16 isolates were selected from a pool of 302 Finnish isolates: half of which were obtained before 2008 and the other half after 2009. These isolates were subjected to qunatification of produced AC enzyme activity concurrently with ACT expression ELISA. The AC enzymatic activity was evaluated by Peter Sebo's laboratory through the measurement of cAMP formation in the presence of calmodulin, as previously outlined by Ladant et al. [19]. Briefly, 10 µl of the bacterial lysate (*B. pertussis* culture at OD 1.0 mixed 1:1 with 8 M urea) was mixed with 50 µl of the AC mix containing radioactive alpha-[^{32}P]-ATP. Enzymatic AC activity was measured in the presence of 1 µM calmodulin [19]. The enzymatic reaction was stopped in time intervals by the addition of 0.5 M HCl, and samples were heated for 5 min at 100 °C. The samples were neutralized by the addition of 1.5 M unbuffered imidazol and the cAMP

was separated from ATP on aluminium oxide columns and eluted elution of [^{32}P]-cAMP by 10 mM imidazol (pH 7.6). The radioactivity in the sample was determined by scintillation counting (Hidex 600 SL). One unit of adenylate cyclase corresponds to 1 μmol of cAMP formed in 1 min at pH 8 at 30 °C. $N = 3$ (in monoplicate).

2.4. Comparison of antigen expression between isolates carrying different serotypes and ptxP genotypes

Groupwise comparison of antigen expression between FIM2/FIM3 and *ptxP1/ptxP3* carrying isolates were performed to evaluate the potential association between FIM serotype or *ptxP* allele and the changes in expression of other studied antigens. The serotypes of isolates were examined as previously described [5,14]. Genotype profiles of isolates were routinely analyzed [11]. For comparison between serotypes, antigen expression of 162 FIM2 and 106 FIM3 carrying isolates were compared. For *ptxP* alleles, antigen expression between 85 *ptxP1* and 155 *ptxP3* were compared. Isolates lacking information on FIM serotype and *ptxP* alleles were excluded from this comparison.

2.5. Statistical analysis

For statistical analysis, non-parametric statistical analyses were used for all antigen expression data, assuming them to be non-parametric variables. To examine possible alterations of antigen expression within the study period, Kruskal-Wallis test was performed. To evaluate difference between groups, Mann-Whitney *U* test was performed.

The groupwise comparison of antigen expression between isolates carrying either FIM2 ($n = 162$) or FIM3 ($n = 106$) and *ptxP1* ($n = 85$) or *ptxP3* ($n = 155$) was performed with Mann-Whitney *U* -test.

The Spearman correlation was utilized to assess the significance of the correlation between the expression of ACT and FHA. Statistical analyses were performed with GraphPad Prism 9.2.0 (GraphPad Software, Inc., Boston, USA) with a two-sided significance level of <0.05 was used.

3. Results

3.1. Validation of the antigen expression measurement assay

The assay was proven to be specific and reproducible as the OD value of negative control was low and CVs of both inter- and intra-assays were low for all antigens tested (Table 3).

The standard curves of the assay for different antigens are shown in Fig. 2. The measured, non-normalized expression level (absorbance) of each isolate and each antigen remained within the linear range, demonstrating that the assay was accurate and capable of detecting potential changes in antigen expression within these interpreted linear ranges.

3.1.1. FHA expression

A significant change was observed in the expression of FHA during the study period of 1991–2020 (Fig. 3), demonstrating a 2.1-fold increase (Mann-Whitney *U* test, $P < 0.0001$), with a rapid 1.75-fold increase noted after the period of 2006–2010 (Mann-Whitney U test, $p < 0.0001$). The expression level of FHA varied from 0.86 AU to 5.71 AU among the tested isolates. None of the 302 isolates analyzed during the period 1991–2020 were identified as FHA deficient.

Table 3
Co-efficient of variation in inter and intra-assays*

	PRN	PT	FHA	ACT
Intra-assay, CV-%	4.0 %	4.6 %	4.9 %	4.9 %
Inter-assay, average CV-% (CV% range of individual assays)	5.1 % (3.3–7.1 %)	5.9 % (5.7–6.1 %)	5.3 % (4.7–5.8 %)	4.0 % (3.0–5.7 %)

* Three strains were used for analyses of inter-assay variation, and for intra-assay variation only 1 strain was used with 5 replicates.

3.1.2. PRN expression

Out of the tested isolates within the study period, 47 (15.6 %) were found to be PRN deficient. First Finnish PRN deficient isolate was detected in 2011 [20]. Within the study period increasing prevalence of PRN deficient isolates was recorded. The annual prevalence of tested PRN-deficient isolates in Finland is presented in Table 2. In addition to the increased proportion of PRN deficient isolates, the expression level of PRN is decreasing with time ($p = 0.032$) among the PRN positive isolates (Fig. 3). However, there was individual variation in expression levels with range from 0.41 AU to 1.87 AU.

3.1.3. PT expression

The expression level of PT varied from 0.39 AU to 2.40 AU among the tested isolates. None was found to be PT deficient. However, alterations in expression of PT among isolates were observed. The expression of PT increased significantly ($p = 0.027$, Kruskal-Wallis) within study period (Fig. 3). Specifically, PT expression showed an increasing trend until the period of 2001–2005, coinciding with the emergence of *ptxP3* alleles. Subsequently, a decline in PT expression was observed until the period of 2011–2015, followed by a notable increase between time periods of 2011–2015 to 2016–2020 ($p < 0.01$, Mann-Whitney *U* -test).

3.2. ACT expression and AC activity

Within the study collection, a statistically significant 1.7-fold increase ($p = 0.0084$, Kruskal-Wallis) in expression of ACT within the study period was observed (Fig. 3). The main increase was observed following 2006–2010, when a rapid 1.5-fold increase in ACT expression was observed, coinciding with an accelerated increase in FHA expression (Mann-Whitney *U* test, $p = 0.0041$). The expression of ACT in individual isolates varied between 0.49 AU and 4.52 AU. All studied isolates expressed ACT. AC activity performed in parallel to ACT expression ELISA revealed over 1.8-fold increase between activities of randomly selected isolates before and after 2009 when 3-component DTaP3 vaccine was used (8 isolates for each period, data not shown). A strong correlation between AC activity and ACT expression of testing isolates was observed ($r = 0.74$, $r^2 = 0.55$).

3.2.1. Alterations in expression of antigens in isolates with different fimbrial serotypes and ptxP alleles

We next wanted to compare if the alteration in antigen expressions was associated with different FIM serotypes and *ptxP* genotypes. Interestingly, the significant difference in the expression of PRN and PT, but not FHA and ACT, was found between isolates with FIM2 ($n = 162$) and FIM3 ($n = 106$) serotypes (Fig. 4). Significantly higher expression of PRN was observed in isolates with FIM2 serotype compared to those with FIM3 serotype ($p < 0.0001$, Mann-Whitney *U* -test). Additionally, a significantly lower expression of PT was linked to isolates with the FIM2 serotype compared to isolates with the FIM3 serotype ($p = 0.0002$, Mann-Whitney *U* test). No significant difference in the expression of FHA or ACT was observed between isolates carrying the FIM2 and FIM3 serotypes.

The same comparison was performed between isolates with *ptxP1* and *ptxP3*, revealing significant differences in expression of all studied antigens (Fig. 5). Among these, isolates with the *ptxP3* allele ($n = 155$) exhibited significantly higher expression of ACT ($p = 0.0033$), FHA ($p < 0.0001$), and PT ($p < 0.0001$) compared to isolates with the *ptxP1* allele ($n = 85$). However, PRN expression in isolates with *ptxP3* allele was significantly lower than those with *ptxP1* allele ($p < 0.0001$).

3.2.2. Correlation between the expressions of FHA and ACT

While examining the values of antigen expression and possible correlations between different antigens, a strong correlation ($r = 0.784$, $p =$

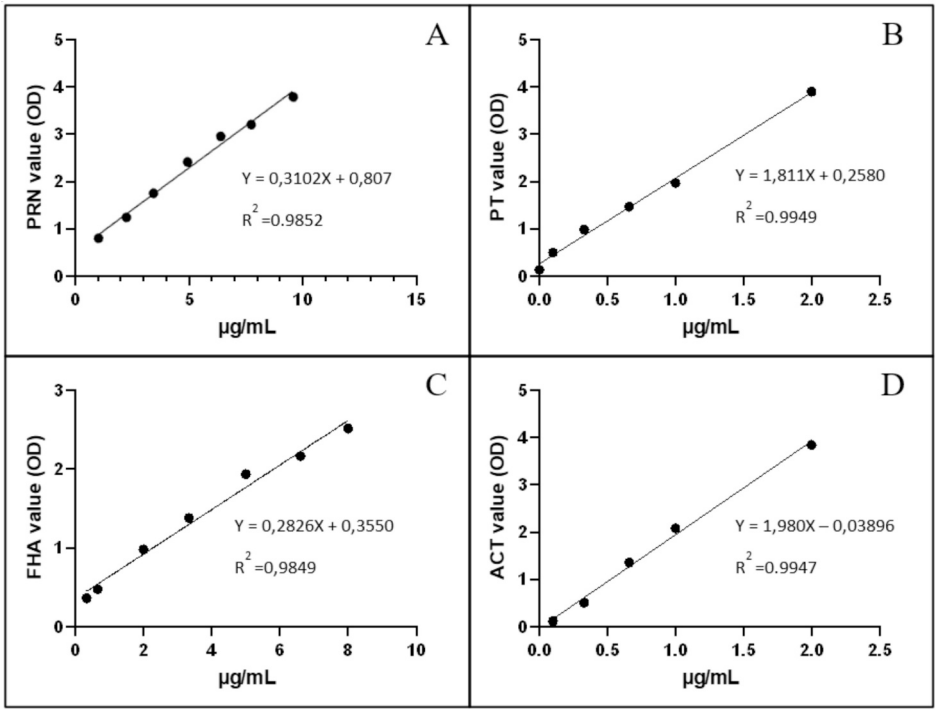

Fig. 2. Standard curves of antigen expression ELISA. Interpreted standard curves for expression-ELISA-assay. Standard curves were interpreted for all antigens measured: PRN (A), PT (B), FHA (C) and ACT (D). OD = Optical density.

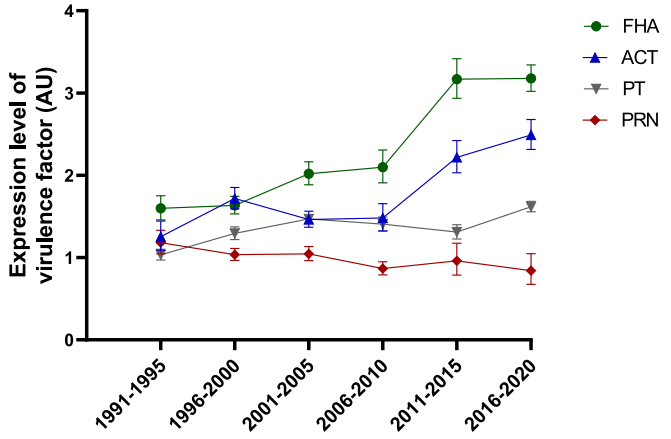

Fig. 3. Alterations in antigen expression level of *B. pertussis* in 1991 to 2020. Median expression levels of Finnish *B. pertussis* isolates antigens, FHA, ACT, PT and PRN in arbitraty units (AU) of each period. All isolates expressed PT, FHA and ACT. In PRN expression only PRN-positive isolates are included. All isolates possess PT, ACT and FHA genes. Number of tested isolates in the periods 1991–1995; 35, 1996–2000; 60, 2001–2005; 61, 2006–2010; 34, 2011–2015;35, and 2016–2020; 77. 95 % confidence intervals presented.

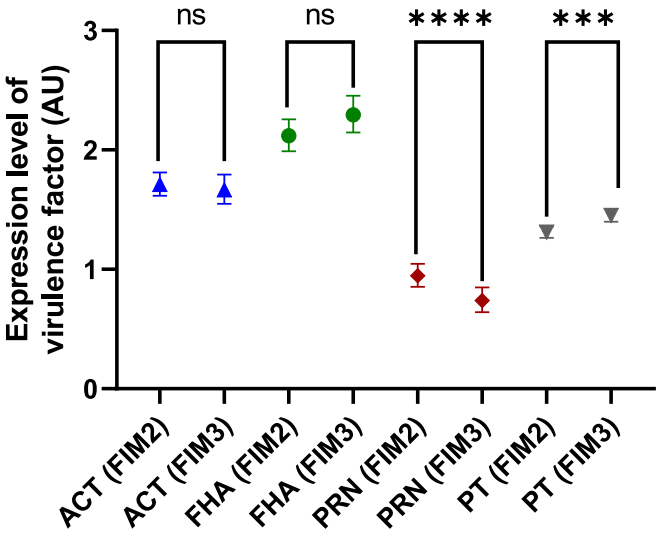

Fig. 4. Alterations between FIM2 and FIM3 serotype isolates in expression of antigens during 1991–2020. Median values (arbitraty units) of each antigen compared between FIM2 and FIM3 isolates. 95 % confidence intervals presented. The statistical tests were performed with Mann-Whitbey *U* test, ns. > 0.05, *** < 0.001, **** < 0.0001.

0.0093 Spearman correlation) between FHA and ACT expression was found in isolates collected after 2005 (Fig. 6). However, no such correlation was observed in isolates collected before 2005. Correlations between expressions of other antigens were not found.

4. Discussion

Significant changes in circulating *B. pertussis* strains occurred shortly after aPvs implementation. These vaccines, being effective, safe, and decreasing the incidence of pertussis, may have also selected and facilitated the emergence of antigenic variant strains adapted better to vaccinated populations, particularly those deficient in PRN [4,9,10,15].

In this study, we aimed to explore alterations in the expression of three antigens, FHA, PT, and PRN, included, and one important antigen, ACT, not included in the current aPvs, among *B. pertussis* isolates collected before and after the introduction of aPv in Finland. To our knowledge, this is the first study to examine the expressions of several vaccine and non-vaccine antigens of *B. pertussis* in an integrated analysis based on a large number of isolates collected during a period of 30 years.

The assay used demonstrated robust specificity and reproducibility, indicated by low CVs for both inter- and intra-assays across all tested antigens. Moreover, the accuracy of the assay was further reinforced

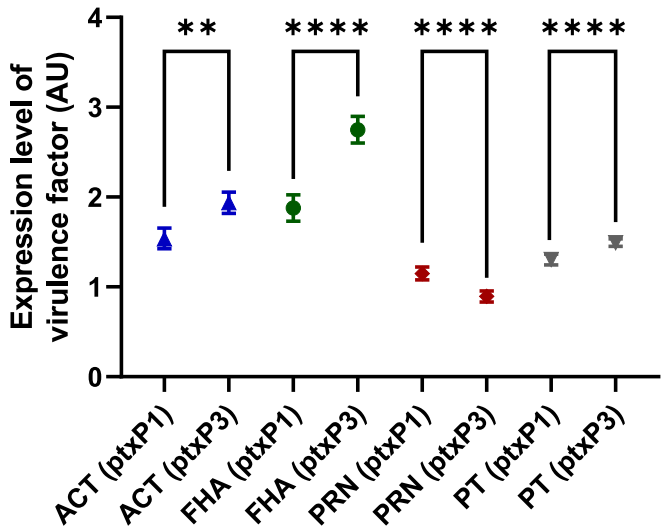

Fig. 5. Alterations between *ptxP1* and *ptxP3* serotype isolates in expression of antigens during 1991–2020. Median values (arbitraty units) of each antigen compared between FIM2 and FIM3 isolates. 95 % confidence intervals presented. The statistical tests were performed with Mann-Whitney U test, ns. > 0.05, ** < 0.01, *** < 0.001, **** < 0.0001.

through the interpretation of standard curves for each measured antigens, demonstrating that the assay remained accurate and capable of detecting potential changes in antigen expression within the linear ranges.

Results of this study suggest that the use of aPv has exerted a selective pressure on circulating *B. pertussis* to modulate its immune evasion tactics. FHA, PT, and ACT are all known to inhibit the host immune response [21–25]. FHA also functions as an adhesin that mediates the attachment of *B. pertussis* to host respiratory epithelium. PRN, on the other hand, is an autotransporter protein that facilitates the invasion and survival of *B. pertussis* in the host cells. However, PRN is also one major target of the host antibody response and a component of most aPvs. Therefore, *B. pertussis* may have evolved to downregulate PRN expression and/or produce PRN variants that escape the vaccine-induced immunity.

The rare number of FHA and PT deficient isolates combined with increased expression level of two antigens, FHA and ACT, shown in this study implies the significance of the three antigens in pathogenesis of *B. pertussis* [26]. The increased expression of both FHA and ACT may partly compensate for the loss of PRN adherence function and confer an advantage to *B. pertussis* in the aPv era. It is known that FHA and ACT synergize to enhance the colonization of *B. pertussis* in the respiratory tract and suppress the host innate and adaptive immunity. Previous study has also shown that FHA and ACT can interact physically and functionally, and that the co-expression of these two factors increases the virulence of *B. pertussis* in animal models [27].

The reported increase in *B. pertussis* ACT expression and ACT activity confirms and reinforces the findings. This result indicates that increased ACT expression may lead to greater pathological effects by enhancing toxin activity. This underlines the possible potential for heightened virulence and immune modulation, signifying a more potent pathogenic effect of *B. pertussis*.

Our findings showed significant differences in both PRN and PT expression between isolates with FIM2 and FIM3 serotypes, as well as between isolates with *ptxP1* and *ptxP3* genotypes. Additionally, we observed a higher expression of ACT, FHA, and PT in isolates with *ptxP3,* while PRN expression was lower in isolates with *ptxP3* compared to those with the *ptxP1* allele. Furthermore, a strong correlation between FHA and ACT expression was found in isolates collected after 2005, suggesting potential co-regulation or interaction between these antigens.

These results are consistent with previous findings that highlights the impact of *ptxP* alleles on toxin production and further the distinct antigenic profiles associated with different serotypes. Previously the higher expression of *fhaB* has been linked with *ptxP3* rather than *ptxP1,* but not directly associated with elevated FHA expression [28]. However, in this study we were able to show an increase in FHA expression over time in *ptxP3* carrying isolates. The observed increase in PT expression in this study might be linked to the emergence of specific *ptxP3* alleles [1]. As observed also in this study the decreased PRN expression in isolates with *ptxP3* is aligned with previous findings [1]. This further underlines the complexity of the interplay between different genotypes and the expression of antigens.

To date, no previous studies have reported on the association between antigen expression and FIM serotypes. Our results suggest an association between FIM serotype and increased expression of PT and PRN might be influenced more by the presence of the *ptxP3* allele rather than the serotype itself, supported by the fact that most of the FIM3 isolates carry *ptxP3* allele rather than *ptxP1* [29].

Whether the loss of PRN is facilitated/compensated by the notably increased expression of ACT and FHA prompts further investigation into the potential mechanisms driving this increase. The grounds for such a rapid surge in the expression of these antigens is likely to be linked with the adaptations of specific genes or regulatory changes within *B. pertussis* isolates. However, this remains undiscovered and requires deeper investigation into the underlying molecular mechanisms contributing to this phenomenon, providing valuable insights into the further understanding, not limited to the factors influencing the

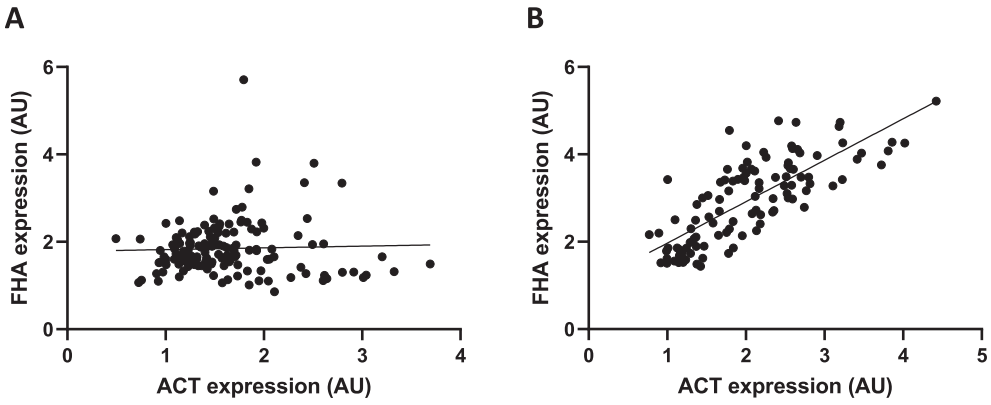

Fig. 6. Correlation between expression of FHA and ACT before and after introduction of acellular pertussis vaccine. A) Correlation between expression of FHA and ACT in isolates collected before 2005 (r2 < 0.01, $r = 0.036$ Spearman correlation). B) Correlation between expression of FHA and ACT in isolates collected after 2005 (r2 = 0.598, $r = 0.784$ $p = 0.0093$ Spearman correlation).

expression of FHA and ACT, but also the potential implications for *B. pertussis* pathogenicity and virulence.

In terms of studying the effects of vaccines on circulating *B. pertussis*, Finland provides unique environment possessing high vaccination coverage accompanied with the consistent use of two-component (PT and FHA) or three-component (PT, FHA, and PRN) acellular vaccines [2,14]. This combined with large number of isolates collected during last 30 years, multiple antigen measurements within isolates and the analyses between phenotypes and genotypes demonstrates the advantage of this study.

5. Conclusions

In conclusion, our study offers valuable insights into the dynamic changes in *B. pertussis* antigen expression over time and their associations with specific genotypes, emphasizing the need for ongoing monitoring of antigenic profiles and genotypic variations in *B. pertussis* to facilitate effective vaccine development and surveillance strategies. Furthermore, our findings contribute to the expanding evidence that *B. pertussis* can adjust its antigen expression in response to the selective pressures imposed by aPvs, demonstrating the bacterium's remarkable capacity for rapid adaptation. These discoveries carry significant implications for comprehending pertussis pathogenesis, transmission, and prevention to validate and expand upon these findings.

Author contribution

QH, JM, and AMB conceptualized the project, and all authors designed the study. VN performed experiments, and JH and JM measured AC activity. VN, AMB, and QH were responsible for data collection, and all authors analyzed and interpreted the data. VN and QH drafted the manuscript, and all authors were involved in critically revising the manuscript for important intellectual content. All authors approved the final manuscript and accepted final responsibility for the decision to submit for publication.

CRediT authorship contribution statement

Vili Niinikoski: Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Data curation. **Alex-Mikael Barkoff:** Writing – review & editing, Methodology, Formal analysis, Data curation, Conceptualization. **Jussi Mertsola:** Writing – review & editing, Methodology, Investigation, Formal analysis. **Jana Holubova:** Writing – review & editing, Methodology, Investigation, Formal analysis, Data curation. **Jiri Masin:** Writing – review & editing, Methodology, Investigation, Formal analysis, Data curation. **Peter Sebo:** Writing – review & editing, Methodology, Investigation, Formal analysis, Data curation. **Qiushui He:** Writing – review & editing, Writing – original draft, Supervision, Methodology, Investigation, Funding acquisition, Formal analysis, Conceptualization.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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