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Resurgence of pertussis and strategies of *B. pertussis* to evade acellular vaccine induced immunity

Faculty of Medicine, Institute of biomedicine
Master's thesis

Author:
Vili Niinikoski

Supervisors:
Alex-Mikael Barkoff, M.sc
Project Researcher
Institute of Biomedicine
University of Turku

Qiushui He, M.D. and Ph.D
Professor
Institute of Biomedicine
University of Turku

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Author(s): Vili Niinikoski
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In countries where acellular pertussis (aP) vaccines are used, there has been a rise in *B. pertussis* isolates that do not produce certain vaccine antigens, such as pertactin (PRN). In Finland, the whole-cell pertussis (wP) vaccine was replaced by aP vaccines in 2005, which contained only two components: pertussis toxin (PT) and filamentous hemagglutinin (FHA). In 2009, the aP vaccines were changed to a three-component vaccine, which included PT, FHA, and PRN. Two years after the change, first PRN-deficient isolate was reported. After that number of PRN-deficient isolates has increased, but no PT or FHA-deficient isolates were found. Whether *B. pertussis* has used different strategy for other aP vaccine antigens remains to be discovered.

The objective of our study was to examine changes in the expression of four antigens, including three (PT, PRN, and FHA) that are present in aP vaccines, and one (adenylate cyclase toxin or ACT) that is not included in current aP vaccines. We analyzed Finnish isolates collected over the past 30 years.

We included three hundred isolates, chosen at random from the Finnish Reference Laboratory for Pertussis and Diphtheria located in Turku, Finland, between 1991 and 2020. We employed an adjusted ELISA-based assay that uses monoclonal antibodies and included culturing *B. pertussis* in Stainer-Scholte medium to assess antigen expression levels. The expression of each antigen by individual isolates was compared using arbitrary units.

Following the implementation of aP vaccines, there has been a substantial rise in the number of PRN deficient isolates, and even PRN positive isolates have shown a decrease in PRN expression, particularly after the use of aP vaccines containing PRN. However, we did not detect any FHA or ACT negative isolates, and the production of PT, FHA, and ACT in the *B. pertussis* strains isolated after 2010 has significantly increased. Nevertheless, only the increase in FHA and ACT expression was correlated with the introduction of aP vaccines.

The study revealed that *B. pertussis* has employed distinct strategies for the production of different antigens in response to selection pressure induced by aP vaccines. After the introduction of aP vaccines in Finland, the production of FHA and ACT increased significantly in *B. pertussis* isolates. These findings highlight the need for further investigation into the production of FHA and ACT in *B. pertussis* strains from countries with varying aP vaccines and immunization programs.

Key words: Pertussis, *B. pertussis*, Resurgence.

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1 Introduction

1.1 Pertussis

1.1.1 History of pertussis

First records of pertussis go back to Sui Dynasty of the seventh century when the pertussis-like disease was described likely by Yuanfang Chao and other Chinese leading medical authorities, as “the cough of 100 days”. (Y. Liang et al., 2016) When earliest recorded epidemics of pertussis were suggested to took place in Persia, the outbreaks in Europe were recorded in 16th century still without recognizing the causative agent behind the disease (Aslanabadi et al., 2015; Cone, 1970). This time description of pertussis in France was provided in 1578 by Guillaume De Baillou. (Cone, 1970) However, the recognition of causative agent of pertussis took 300 hundred more years. In the end of 19th century one of the first to recognize the gram-negative bacterial rods causing pertussis from sputum specimen from clinical pertussis was Belgian scientist Jules Bordet. Still the isolation of the causative agent of pertussis remained out of reach until 1906 when *B. pertussis* was successfully isolated by Jules Bordet and Octave Gengou. (Oakley, 1962) Jules Bordet was then awarded with 1919 Nobel prize in Physiology or Medicine namely for his work on the complement system, but also antimicrobial field including the study of *B. pertussis* and its identification in pertussis disease (The Nobel Prize in Physiology or Medicine 1919. NobelPrize.Org. Nobel Prize Outreach AB, 2024).

Despite of the recognition of the causative agent of pertussis, it remained as a major cause of morbidity and mortality among children until the introduction of first vaccine to prevent pertussis. In the 1950s the first whole-cell vaccine against pertussis (wP) was globally implemented and incidence of pertussis were significantly decreased (Figure 1). Despite of vaccines, pertussis still circulates in the population. (Centers for Disease Control and Prevention (CDC), 2022; Lauria AM, 2015)

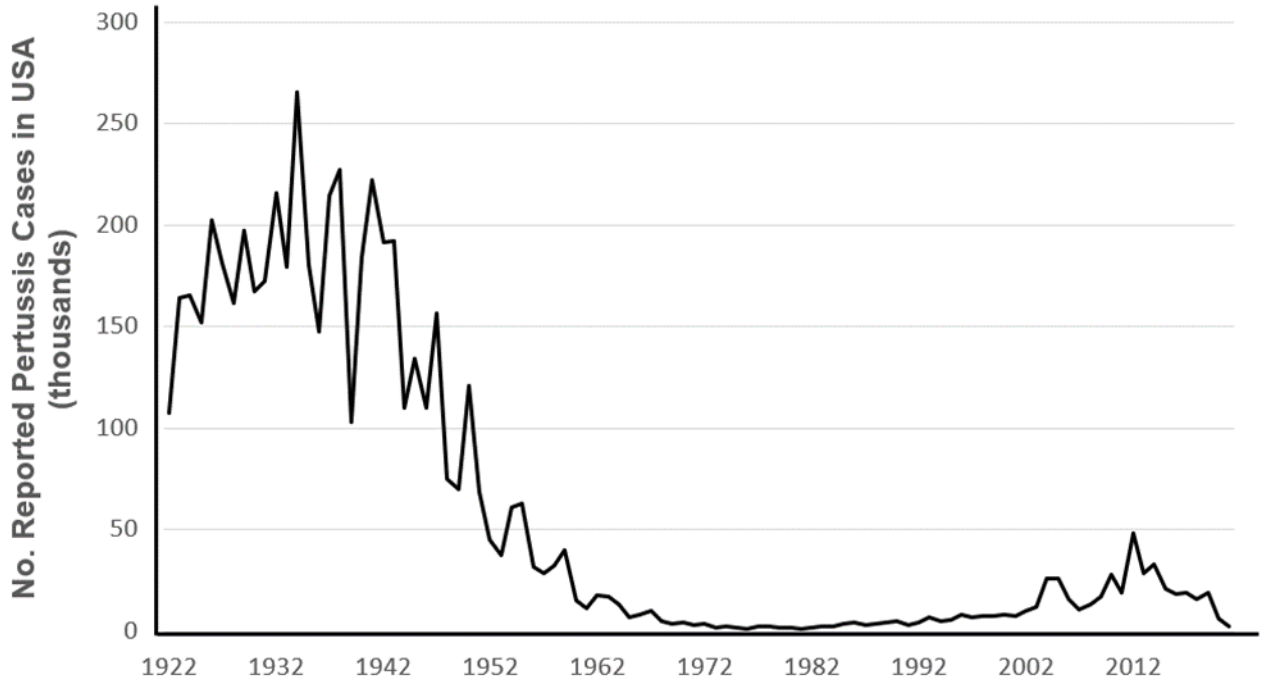

Figure 1. Reported pertussis cases in the United States from 1922 to 2021. Data source: Centers for Disease Control and Prevention (CDC), 2022). Figure created by Vili Niinikoski.

1.1.2 Epidemiology

The number of cases has decreased from the pre-vaccine era up to 95% to this date due to introduction of pertussis vaccines. (Figure 1) Back then, pertussis was known to be one of the leading causes of morbidity and mortality among children and infants. Despite of the high vaccine coverages, pertussis occurs worldwide infecting estimated 48,5 million people worldwide with mortality rate slightly below 300,000 annually taken into account that only 5-10% of cases are diagnosed according to Lutwick & Preis, 2014. This is due to atypical, asymptomatic presentation of the disease where the symptoms are mild or totally lacking and help seeking is reduced. The pertussis epidemics reoccur in 3-to-5-year cycles without complying seasonal pattern. (Broutin et al., 2010)

Previously recognized only as childhood disease, pertussis epidemiology has shifted to stage where it affects increasingly also on adolescence and adults. (Güriş et al., 1999) As a highly contagious respiratory infection of which transmission majorly occurs from person to person via respiratory droplets or secretions, the secondary attack rates rise, up to 80% within households. (Warfel et al., 2012; Wirsing von König et al., 1995) The contagiousness of diseases is examined through the basic reproduction number (R0) which expects the number of

cases, that are directly generated by the infected individual in the population to others that are susceptible to infection. The R0-value for pertussis 12 to 17, calculated in the early 20th century is particularly high for example when compared to the R0-value of Covid-19 1.5 to 6.68 based on 12 studies (Anderson & May, 1982; Liu et al., 2020) By using asymptomatic carriers as a reservoir, pertussis promotes the vulnerable population to acquire the severe disease. There are several explanations for the shift in the epidemiology of the pertussis, including early waning immunity and immune evasion of *B. pertussis* afforded by acellular pertussis (aP) vaccines. Despite of vaccines and decreased prevalence, Pertussis is still recognized as cause of morbidity and mortality especially among younger population without immunization. (Mooi et al., 2009)

Similarly, as in elsewhere in the world the incidence and prevalence of pertussis decreased heavily after the implementation of the wP vaccine introduced in 1952 in Finland. The incidence in Finland reached its nadir in 1978, when 10 cases with not a single death was reported. The epidemics of pertussis between 1952- 2004 occurred in a similar cyclic manner as seen in global pertussis reports. The peak of pertussis incidence took place in 2004 during the great epidemics resulting 1631 reported cases. After the epidemic in 2004 and the introduction of aP vaccines the incidence of pertussis has remained stable, and signs of resurgence are yet not to be seen (Figure 2). (Finnish Institute for Health and Welfare (THL), 2024)

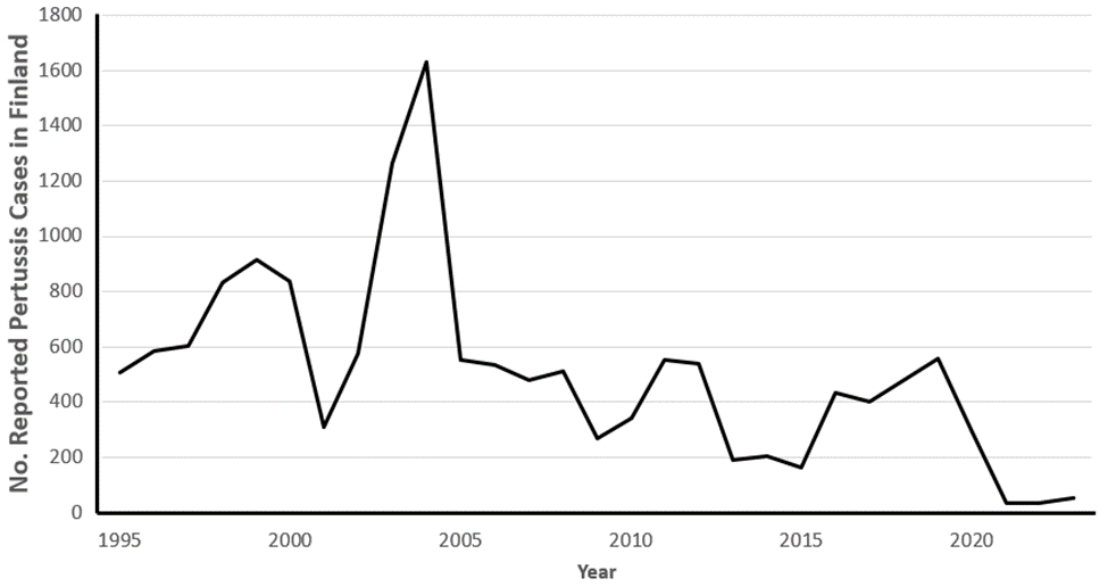

Figure 2. Reported Pertussis Cases in Finland from 1995 to 2022. Data source: Finnish Institute for Health and Welfare (THL), 2024. Figure created by Vili Niinikoski.

1.1.3 Resurgence of pertussis

After a drastic decrease in the incidence of pertussis reported after the introduction of wP vaccines the shift towards increasing incidence has been reported after implementation of aP vaccines. This phenomenon is referred as the resurgence or re-emergence of pertussis. Despite of high vaccine coverages, pertussis has become the most prevalent vaccine-preventable disease in industrialised countries. (de Melker et al., 2000) Theories underlying resurgence of pertussis include the waning vaccine-induced immunity, decreased vaccination coverage, better diagnostics, suboptimal vaccines, and the adaptation of the pathogen (Damron et al., 2020; He & Mertsola, 2008; Mooi et al., 2014; Rachlin et al., 2022). The relative truth behind the resurgence remains unknown and under debate. Though, pathogen adaptation is supported by different observations described later in this thesis (See chapters 1.2.6 to 1.2.9). However, the contribution of these factors is country dependent and hard to generalize due to different practices such as diagnosis, vaccines, vaccination schedules, and coverage (European Centre for Disease Prevention and Control ©, 2024; He et al., 2012; Plans-Rubió, 2021). This thesis focuses on the ability of *B. pertussis* to adapt and survive in the populations with aP-vaccine induced immunity.

1.1.4 Clinical Manifestation

Pertussis is divided to three clinically different stages: catarrhal, paroxysmal, and convalescent (Figure 3). The usual incubation period of whooping cough is seven to ten days with a range between four to 21 days. (Havers et al., 2021) The first stage of the disease is catarrhal stage. In this stage the symptoms such as low-grade fever, runny nose, sneezing and mild occasional cough are observed. The fever remains low throughout the illness. Consequently, the clinical common cold-like symptoms make early diagnosis of the disease difficult. However, after one to two weeks the gradually progressive cough leads to the second, paroxysmal stage of the illness. (Fry et al., 2021; Havers et al., 2021)

In the paroxysmal stage suspicions of whooping cough arise. The characteristic symptoms of whooping cough are linked to this stage including rapid bursts or paroxysms of cough followed by a long inspiratory effort combined with characteristic high-pitched whoop, after which the disease is named. These attacks occur in average of 15 in day being more prevalent at night. Consequently, long lasting cough and difficulties in breathing may result as cyanosis.

Particularly, young infants and children might appear with worse clinical symptoms. However, between attacks the patients' symptoms might appear mild or non-existent. Paroxysmal stage last 1 to 6 weeks with possibility to persist up to 10 weeks. (Fry et al., 2021; Havers et al., 2021)

The third and last stage of whooping cough, convalescent stage last 3 to 4 weeks with gradual recovery. The paroxysms subside in 2 to 3 weeks. However, recurrence of the paroxysms with subsequent infections in respiratory track after the onset of whooping cough is common. (Fry et al., 2021; Havers et al., 2021)

These clinical symptoms presented above might vary due to patient age or vaccination status resulting more severe in younger and unimmunised people including pertussis-related death, commonly caused by secondary bacterial pneumonia. Alternatively, disease may be asymptomatic or milder. In addition, the characteristic high-pitched whoop might be absent. However, the people with asymptomatic or milder disease are capable of transmit the disease. (Fry et al., 2021)

1.1.5 Diagnosis

Pertussis-like symptoms are caused by multiple different causative agents including other *B. pertussis* species, other bacteria such as *Mycoplasma pneumoniae*, *Chlamydia pneumoniae*, and *Mycobacterium tuberculosis* and viruses like adenovirus, respiratory syncytial virus and bocavirus (Frumkin, 2013; Nieves & Heininger, 2016). Further, prolonged non-paroxysmal cough seen in pertussis can also be caused by asthma, sinusitis, cystic fibrosis, bacterial pneumonia (Nieves & Heininger, 2016). To administer adequate treatment, the correct diagnosis plays key role. In pertussis, it is important to diagnose the disease accurately in manner of time to be able to offer treatment to alleviate the symptoms and prevent transmission to the close contacts and further offer prophylactic treatment for vulnerable population to avoid possible fatalities.

As the *B. pertussis* is known to colonize ciliated epithelial cells of the nasopharynx, the isolation of pertussis is obtained from the nasopharynx of the host through nasopharyngeal swaps (NPS) or -aspirates (NPA). In severe disease, pertussis is also reported to colonize lower respiratory track as a result of which isolation through throat swaps is reported to be suboptimal. (Paddock et al., 2008) In infants, neonates, and small children, the isolation from NPA is preferred, and higher isolation rate is obtained. On the contrary, NPSs are preferred by the medical staff for

the adolescent and adult populations. For immunologic diagnose, blood sampling and serum of a blood is needed. (He et al., 2022)

The current methods to diagnose pertussis infections recommended by the European Centre for Disease Prevention and Control (ECDC) include, bacterial culture, polymerase chain reaction (PCR), and serological assays such as enzyme linked immune sorbent assay and multiplexed immunoassays. (He et al., 2022)

As mentioned previously, timely manner is important when diagnosing pertussis and the accurate diagnostic method should be chosen according to patient symptoms and disease progression which makes the diagnosing challenging. The diagnostic sensitivity of different methods varies during the progression of the disease and guidelines for accurate use of these methods are recommended by ECDC (Figure 3) (Fry et al., 2021; He et al., 2022).

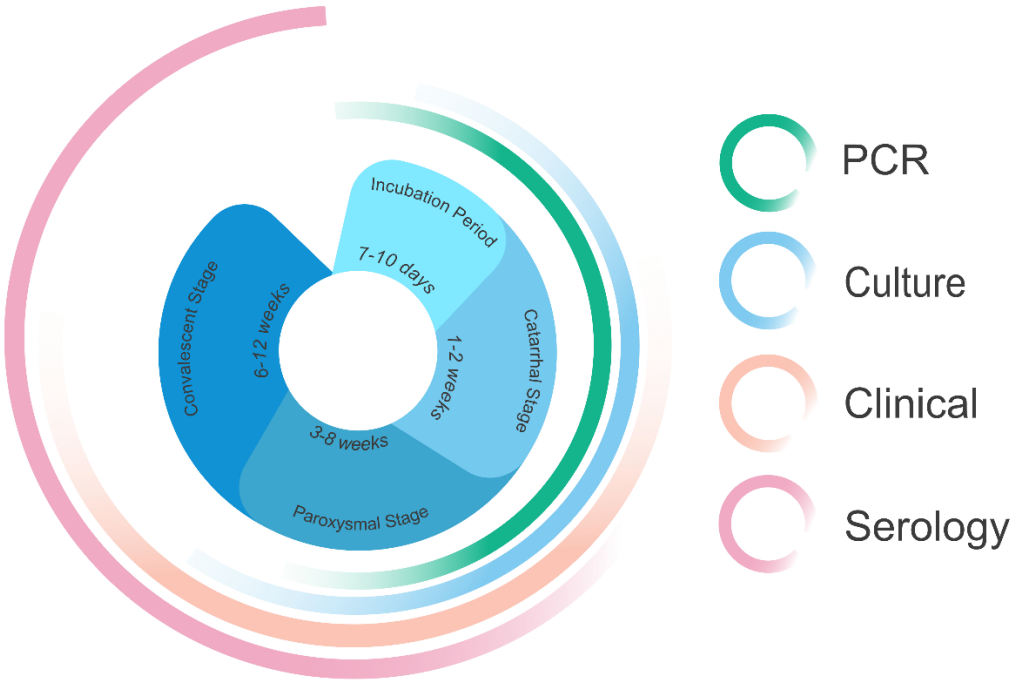

Figure 3. The various stages of pertussis infection and the diagnostic sensitivities of laboratory techniques in different stages of pertussis disease. PCR (green), culture (blue), clinical diagnosis (orange), and serology/oral fluid (red). Figure created by Vili Niinikoski based on Fry et al., 2021).

1.1.6 Vaccination

During the time before vaccination, pertussis was considered as childhood disease being the one of the major mortality causes among the young population. In 1910 in US, 1 out of 10 infected children resulted eventually death (Kuchar et al., 2016). The first wP vaccine against

pertussis was efficient, drastically reducing the incidence of pertussis up to more than 95% (Cherry, 1984). The efficacy of the vaccine was 60-90% and it was dependent on the number of inactivated bacteria in the vaccine. (Onorato et al., 1992) However, increase of the amount of bacteria in the vaccine was discouraged due to possible increased toxicity of the vaccine. The usage of the wP vaccines was associated with increased incidence of severe neurological disease, vaccine encephalopathy (J. M. Berg, 1958; Kulenkampff et al., 1974). Awareness of the possible adverse reactions led to decrease in the use of wP vaccines and even a discontinuation in some countries (Kulenkampff et al., 1974). However, this possible association between wP vaccines and vaccine encephalopathy was proven false in the later studies (Cowan et al., 1993; Miller & Ross, 1978; Ray et al., 2006).

The first steps towards cell-free vaccines were made in 1954 when Pillemer developed an early version of aP vaccine (Armitage et al., 1956;Council, M. R. 1956). However, the first aP vaccines were developed in Japan in 1980s to be less reactive alternative for wPs (Sato et al., 1984; Schmitt-Grohé et al., 1997). First aP vaccines were licenced for infant use in industrialized countries in early nineties. This was followed by transition phase where aP vaccines were used as booster vaccinations after wP priming. However, aP vaccines gradually replaced wP vaccines in industrialized countries as also in Finland the wP vaccine was replaced by aP in 2005. (Guiso et al., 2020; Versteegen et al., 2021) The aP vaccines developed to this date contains one to five highly purified *B. pertussis* antigen components; PT, FHA, PRN, fimbria (FIM) serotypes two (FIM2) and three (FIM3) (J. L. Liang et al., 2018) In contrast, the number of antigens in the wP vaccine is significantly higher including thousands of antigens. Despite of number of vaccine components, exclusive testing of aP vaccines in the 1990s revealed the exceeded efficacy and safety of aP vaccines compared to the wP vaccines (Edwards et al., 1995) The multiple different versions of aP vaccines containing one to five components most commonly includes PT specific for *B. pertussis* and highly immunogenic FHA. Mono- (only PT), bi- (PT and FHA) component vaccines remain same but alterations in multicomponent vaccine occur. However, most commonly including PT, FHA, and PRN or all the 5 components listed above (Edwards et al., 1995; Taranger et al., 2001; Vidor & Plotkin, 2008).

1.1.7 Current vaccination

To reach the optimal protection against the disease, the full immunization including primary and booster series are necessary. Currently both aP and wP vaccines are used. The aP vaccines are commonly used in developed countries whereas the wP vaccines are more commonly used in the low- and middle-income countries. (Sealey et al., 2016) The formulation of an aP vaccines vary depending on manufacturer. For example, the US Food & Drug Administration has granted approval to several pertussis vaccines. Among them, seven distinct DTaP vaccines are recommended for pediatric usage, while only two Tdap vaccines containing tetanus toxoid with reduced diphtheria toxoid and acellular pertussis are available for booster shots. (U.S. Food & Drug Administration, 2023) Likewise, the European Medicines Agency has authorized various DTaP and Tdap vaccines, but the accessibility differs among member states(European Medicines Agency, 2021). Current pertussis vaccines are commonly combined with diphtheria-, tetanus toxoids or other disease components (European Medicines Agency, 2021; U.S. Food & Drug Administration, 2023)

For vaccination schedule of pertussis there are different recommendations. World Health Organization (WHO) has its own recommendations for diphtheria-tetanus-pertussis-containing vaccines. The primary series of these vaccines initiating as early as six weeks after birth and to be continued with subsequent doses at 10-14 and 14-18 weeks. In addition to the primary series, the boosters are recommended by WHO, especially at two years of age. (WHO, 2016) Whilst, ECDC recommendations include use of either DTaP or DTwP for the three-dose primary immunization given at 2-12 months of age with aP booster doses at 1 to 2 years and at 3-6 years. However, authority recommendations serve as a basis and inter-country variations of the vaccination schedule exists. (European Centre for Disease Prevention and Control ©, 2024)

Unfortunately, the population at the highest risk of the pertussis are infants under two months of age who are too young to be fully vaccinated (Winter et al., 2012). However, various strategies to protect this vulnerable population are employed. The more potent alternative lies on maternal immunization where pertussis specific IgG antibodies transfer to the foetus through transplacental transfer. (Gall et al., 2011) This is proven to provide high protection against pertussis prior to primary immunization (Amirthalingam et al., 2016). Therefore, the maternal immunization against pertussis during 2nd and 3rd trimesters is recommended by Centres for Disease Control and Prevention (CDC) regardless of the vaccination status of the mother (Centers for Disease Control and Prevention (CDC), 2013). Maternal immunization against

pertussis is routinely recommended in many countries. However, maternal antibodies have been reported to potentially interfere the optimal effect of routine immunization. This phenomenon called ¨blunting effect¨ have risen questions of the negative effects of the maternal immunization and raised the concerns of suboptimal protection after first doses and possibly increases the burden of the disease. (Kandeil et al., 2020) Other strategy to protect the vulnerable population is cocooning where the people in close contact with the vulnerable individual are immunized to prevent possible transmission. However, cocooning is proved to be extremely difficult to implement in practice, in addition it is resource intensive, and the efficacy is questionable. (Skowronski et al., 2012)

1.1.8 Pertussis vaccination in Finland

The pertussis vaccinations in Finland were implemented to the national immunization protocol (NIP) in 1952 (Mertsola et al., 1982). That time the primary series included doses of 3, 4, 5, and 24 months of age, supplemented with booster at 6 years of age. The first acellular booster vaccine was implemented to the NIP in 2003, when the 6-year wP booster was replaced with acellular booster (Boostrix™, GSK). Later in 2005 the aP priming was first time introduced in Finland, and the wP vaccines was totally replaced with aP vaccines (Pentavac™, Sanofi) containing two vaccine components, PT and FHA. Simultaneously the vaccination schedule was changed, and the primary series were dosed at 3, 5, and 12 months of age. This time booster was given at age 4 (Tetravac™, Sanofi), and between 14-15 years (Boostrix™, GSK). In 2009, the Pentavac™ priming series was changed to the Infantrix™ (GSK), containing three pertussis vaccine components, PT, PRN, and FHA. One booster dose (Boostrix™, GSK) was further added in 2012 to be dosed during military service and another in 2018 as a booster for adults including population excluded from military training. However, in 2019, the three-component (Infantrix, GSK) vaccine was switched back to the two-component vaccine (Pentavac™, Sanofi) remaining the current vaccine used in Finland. (Versteegen et al., 2021)

1.2 B. pertussis

The sole agents causing pertussis are the *B. pertussis* and *B. parapertussis* (European Centre for Disease Prevention and Control, 2023). Though, *B. pertussis* remains clearly the main

causative agent causing pertussis. However, also other *B. pertussis* species can cause symptoms that resembles whooping cough i.e. *B. holmesii* and rarely *B. bronchiseptica* (Gupta et al., 2019; Mooi et al., 2012).

As the main causative agent of pertussis in humans, *B. pertussis* is a human-specific, Gram-negative, aerobic *coccobacilli* with pleomorphic properties. This allows *B. pertussis* to adapt to changing conditions in vaccine induced immunity. These adaptations can include changes in morphology, biological functions, or reproductive modes. Evidence of *B. pertussis* pleomorphism have been recorded as an alteration in production of many virulence factors, which are necessary for bacteria to infect eukaryotic host. (Brookes et al., 2018; Mooi et al., 2009; Stefanelli et al., 2006)

B. pertussis produces many proteins which are biologically active or have antigenic properties (Mattoo & Cherry, 2005). Proteins which are necessary for bacteria to infect eukaryotic hosts are called virulence factors. Among the extensively studied virulence factors of *B. pertussis* are three adhesins FIM, PRN, and FHA and two toxins, PT and adenylate cyclase toxin (ACT). Notably, four of these factors have been incorporated as components of aP vaccines, excluding ACT (J. L. Liang et al., 2018).

1.2.1 Pathogenesis and virulence factors

Pertussis is a respiratory disease caused primarily by toxins produced by *B. pertussis*. However, the infection is initiated by its adhesins, virulence factors through which bacteria adheres to the cilia of the respiratory epithelial cells of the host. Thereafter, paralysis of cilia and inflammation of the respiratory track are caused by toxins. This leads to interference in the clearance of pulmonary secretions. In addition to adherence and ability to cause inflammation, the tissue invasion and host defence evasion through virulence factors have recorded. (Havers et al., 2021) In this chapter we cover four virulence factors of *B. pertussis*, two adhesins and two toxins.

1.2.1.1 Pertactin

Pertactin is a β-helical highly immunogenic adhesin produced by most of the *B. pertussis* genus bacteria causing pertussis in humans, for example *B. pertussis*, *B. parapertussis*, and *B.*

bronchiseptica. Due to its high immunogenicity, it is commonly used as a component in aP vaccines. (Edwards & Decker, 2013)

PRN is an autotransporter surface protein which consist of three functional domains: C-terminal autotransporter domain, the passenger domain, and N-terminal signal sequence. The N-terminal signal sequence guides the C-terminal autotransporter domain and the passenger domain to the periplasm of the bacteria, where the autotransporter domain is able to form pore in the outer membrane and translocate the passenger domain to the cell surface. After translocation, the 93kDa precursor protein undergoes proteolytic cleavage to form 69kDa noncovalently attached surface protein. (Edwards & Decker, 2013)

Pertactin is a bacterial adhesin known to mediate adhesion to eukaryotic cells through its arg-gly-asp (RGD) motif. However, the importance of PRN in bacterial adhesion have been questioned, and it is suggested to aid only in the early phase attachment of the infection. (Nicholson et al., 2009; B. M. van den Berg et al., 1999) The persistent attachment is mediated through other adhesins such as FHA and FIM. In addition to adhesion, pertactin is recorded to contribute to transmission, shedding, inhibition of immune protection, and increasing inflammation in nasal cavity (Inatsuka et al., 2010; Ma et al., 2021).

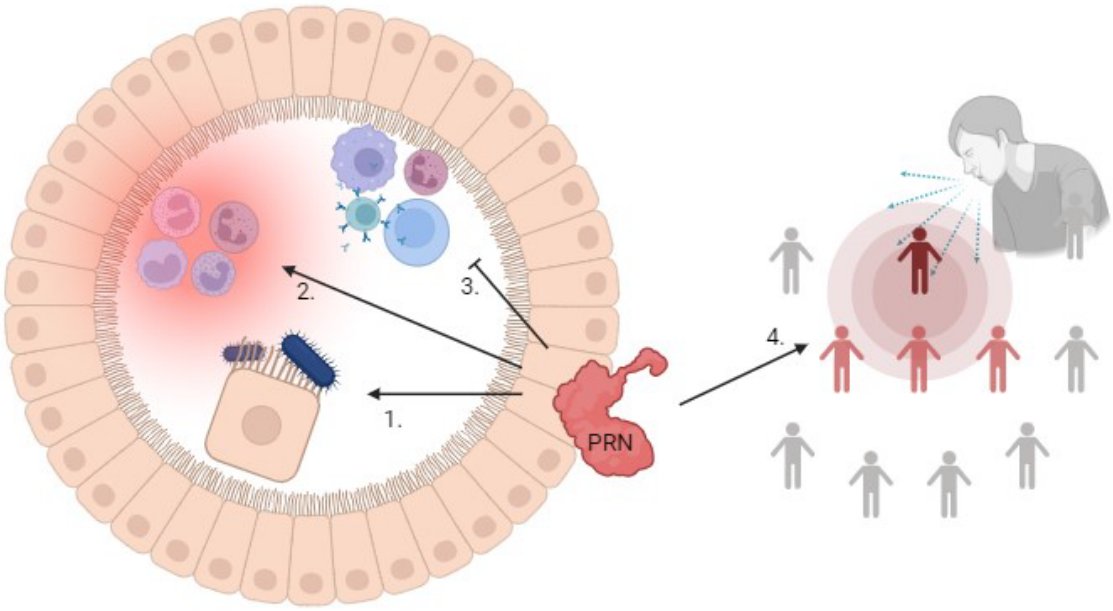

Figure 4. Contribution of pertactin to *B. pertussis* pathogenesis. Pertactin 1.) acts as an adhesin, 2.) increases inflammation in nasal cavity, 3.) hampers immune system function, and 4.) contributes to increased shedding and transmission. This figure is created by Vili Niinikoski with Biorender.com.

1.2.1.2 Pertussis toxin

PT is a specific virulence factor of *B. pertussis*. It plays a central role in pathogenesis of the disease. It is a promoting factor of lymphocytosis and leucocytosis and therefore related to high mortality in young infants. As a unique virulence factor of *B. pertussis,* it is used component of aP vaccines. (Bouchez et al., 2009; Hinds et al., 1996; J. L. Liang et al., 2018; Seitz, 1925)

As an 105kDa multisubunit toxin, PT consists of one enzymatically active subunit A (S1) and of pentamer-binded B-subunits responsible for binding cell surfaces. There are 4 different B-subunits, S2, S3, S4 and S5 of which PT consists of one of each S1, S2, S3, S5 and of two S4 subunits. (Stein et al., 1994).

As an exotoxin PT is secreted by *B. pertussis* via a type IV secretion system (Shrivastava & Miller, 2009). It has been recorded to bind on glycoconjugate molecules on mammalian cells but a PT-spesific receptors have not been identified (Finck-Barbançon & Barbieri, 1996; Witvliet et al., 1989). After endocytosis it is transported to the endoplasmic reticulum via retrograde pathway from where it is thought to translocate to the cell cytosol and activated (Teter, 2019).

In the cytosol ADP-ribosylating S1-subunit of PT is recorded to have inhibitory effects on inhibitory G-protein coupled receptors (GPCR) by modifying the C-terminus cysteine residue of the alpha subunit of G-proteins (el Bayâ et al., 1997; Sakari et al., 2022). This inhibition mediates the disruption of cellular metabolism and is referred as the origin of the systemic pathological effects of *B. pertussis* infection and the cause of lethality (Bouchez et al., 2009; K. M. Scanlon et al., 2017). The effects of PT are target-cell dependent and cause wide–range effects including e.g., islet activation, histamine sensitization and lymphocytosis in animal models (Hinds et al., 1996; Nogimori et al., 1986; K. M. Scanlon et al., 2017). PT also hinders the neutrophil recruitment and macrophage activity in the infection site and reportedly promotes the colonization of host respiratory track (Carbonetti et al., 2005, 2007; Connelly et al., 2012).

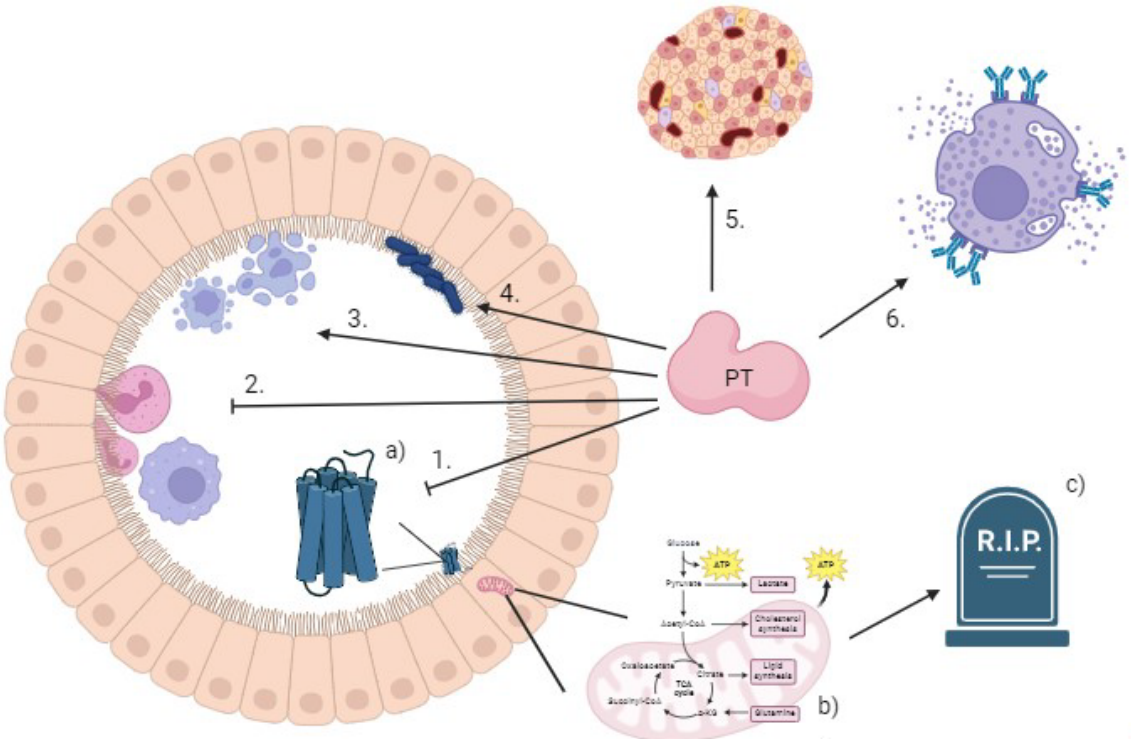

Figure 5. Contribution of pertussis toxin to *B. pertussis* pathogenesis. Pertussis toxin plays a key role in *B. pertussis* pathogenesis by 1a.) inhibiting the effects on inhibitory G-protein coupled receptors and thus 1b.) disrupting of cellular metabolism, causing pathological effects of *B. pertussis* and 1c.) lethality, 2.) hindering the neutrophil recruitment and macrophage activity in the infection site, 3.) causing lymphocytosis, 4.) promoting the colonization of host respiratory track, 5.) Activating Langerhans islets and 6.) causing histamine sensitization. This figure is created by Vili Niinikoski with Biorender.com.

1.2.1.3 Filamentous hemagglutinin

Like PRN, FHA is β-helical, highly immunogenic adhesin used as a primary component of aP vaccines. FHA is surface-associated and secreted virulence factor (Jacob-Dubuisson et al., 2000; Sato & Sato, 1999). FHA produced by *B. pertussis* is one of the most efficiently secreted proteins of gram-negative bacteria. The secretion efficacy alters between the species of *Bordetellae* (Clantin et al., 2004; Jacob-Dubuisson et al., 2000).

The biogenesis of FHA consists of complex processes and the translocation to the bacterial cell surface. It is derived from 367kDa precursor protein proFhaB encoded by *fhaB* gene by proteolytic cleavage of C-terminal 150kDa region (Domenighini et al., 1990). This C-terminal region has proposed to prevent premature folding of the protein before it is exported through cytoplasmic membrane by secretory system to the bacterial cell surface via two-partner

secretion (Tps) pathway (Chevalier et al., 2004; Renauld-Mongénie et al., 1996). This secretion pathway is broadly used mechanism of Gram-negative bacteria to export large β-helical proteins to the cell surface (Jacob-Dubuisson et al., 2013). While exporting FHA, the pathway consists of TpsA passenger protein FhaB and β-barrel pore forming TpsB protein FhaC which transport the proFhaB to the cell surface where it undergoes the proteolytic cleavage to form mature FHA protein (Domenighini et al., 1990; Jacob-Dubuisson et al., 2001). On the cell surface it may remain intact or be secreted to the extracellular space (Domenighini et al., 1990; Sato & Sato, 1999). However, the importance of the secretion and the secretion mechanism of FHA remains unknown (Scheller & Cotter, 2015).

FHA has been proposed to function as both a primary adhesin, an immunomodulator of *B. pertussis* and initiator of the pathogenic cycle of pertussis (Inatsuka et al., 2005; E. I. Tuomanen & Hendley, 1983). The different domains of this large adhesion protein encompass multiple binding capabilities. It is known to mediate attachment on phagocytes via integrin mediated attachment, sulphated sugars of epithelial cells and extracellular matrix via lectin-like binding, and most importantly to the ciliated cells of respiratory epithelium via carbohydrate recognition domain (Smith et al., 2001; E. Tuomanen & Weiss, 1985).

Besides the adherence properties, FHA is known to modulate the innate and adaptive immune system. It is known to trigger the NF-κβ mediated inflammatory responses such as recruitment of inflammatory cells to the inflammation site. However, prolonged exposure of FHA on macrophages was associated with suppression of NF-κβ pathway response to other inflammatory signals. (Abramson et al., 2001; X. Li et al., 2014) FHA is known to modulate immune responses by inhibiting the classical complement activation and T-cell response as well as down-regulating cytokine production (Berggård et al., 1997; Higgs et al., 2012; McGuirk et al., 2002; McGuirk & Mills, 2000). By binding to the macrophages and dendritic cells FHA increases the secretion of anti-inflammatory cytokine IL-10, suppresses Th1 and Th17 response and promotes in the production of IL-10-secreting type 1 regulatory T cells (Higgins et al., 2003; Higgs et al., 2012; McGuirk et al., 2002; McGuirk & Mills, 2000). Increase in IL-10 then again leads to down-regulation of the IL-12 production which have resulted in suppressive effects on macrophages and dendritic cells in murine models (McGuirk & Mills, 2000). Additionally, FHA interaction is reported to supress the neutrophil recruitment, antigen dependent CD4+ T cell proliferation, IL-17 inflammatory mediated response, and to induce the pro-apoptotic responses in human bronchial epithelial and monocyte-like cells (Abramson et al., 2001; Henderson et al., 2012). Also, hyperinflammatory properties such as increased

inflammatory cell infiltration of FHA deficient isolates are reported due to lack of immunomodulatory effects of FHA on host cells (Henderson et al., 2012; Inatsuka et al., 2005). Additionally, FHA mediates biofilm formation for persistent infection and interacts with ACT at cell surface for pathogenesis (Cattelan et al., 2017; Zaretzky et al., 2002).

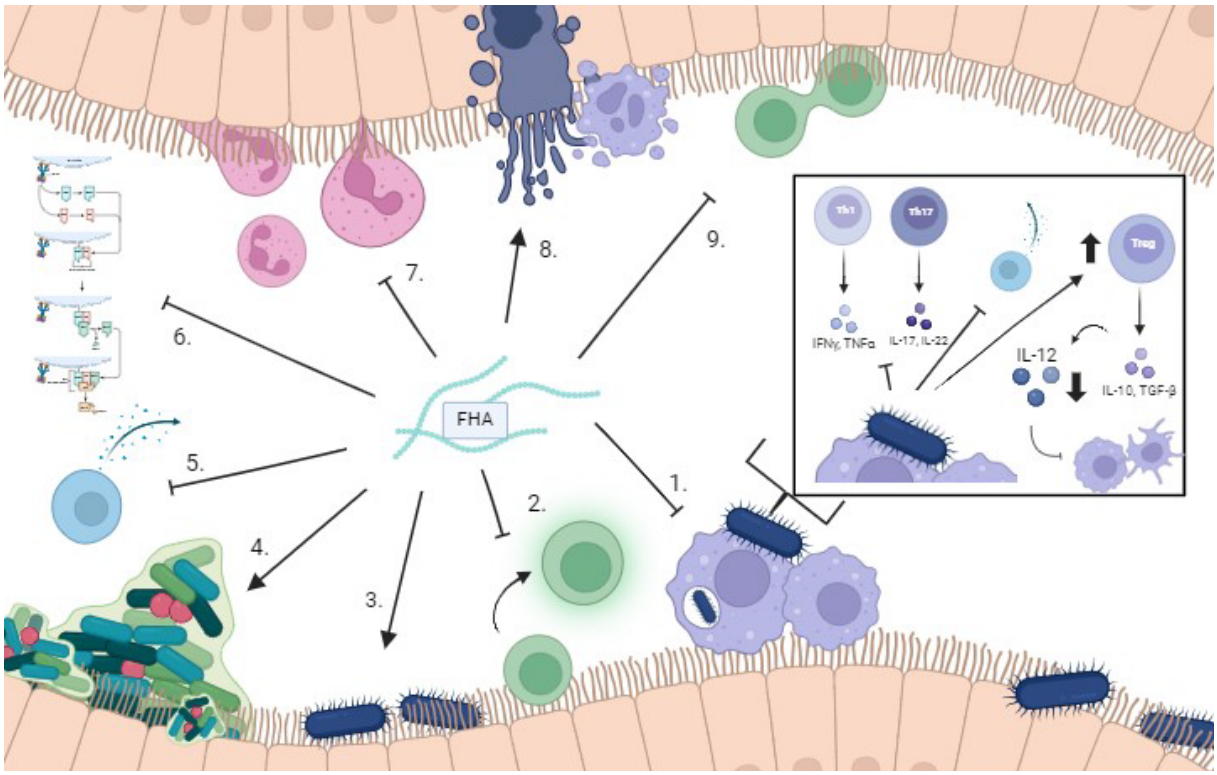

Figure 6. Contribution of filamentous hemagglutinin to *B. pertussis* pathogenesis. Filamentous hemagglutinin: 1.) Increases the secretion of anti-inflammatory cytokine IL-10, suppresses Th1 and Th17 response and promotes in the production of IL-10-secreting type 1 regulatory T cells and confers resistance to *B. pertussis* by inhibiting inflammatory pathology by binding to the macrophages and dendritic cells. 2.) Inhibits T-cell response. 3.) Acts as a primary adhesin. 4.) Mediates biofilm formation. 5.) Down-regulates cytokine production. 6.) inhibits the classical complement activation. 7.) supresses neutrophil recruitment. 8.) induces the pro-apoptotic responses in human bronchial epithelial and monocyte-like cells. 9.) supresses antigen dependent CD4+ T cell proliferation. The figure is created by Vili Niinikoski with Biorender.com.

1.2.1.4 Adenylate cyclase toxin

Along with pertussis toxin, adenylate cyclase toxin is a key toxin for the pathogenesis of *B. pertussis* (Carbonetti, 2010). It is a multidomain toxin with calculated molecular mass of 170kDa which consists of N-terminal adenylate cyclase enzyme domain (ACD), C-terminal

pore-forming repeats in toxin (RTX) hemolysin (Hly) domain, translocation region (TR), hydrophobic region (HR) and acylation region (AR) (Benz et al., 1994; Glaser, Ladant, et al., 1988; Sebo & Ladant, 1993). Despite of its immunogenic properties it has not been included as a component of aP vaccines (J. L. Liang et al., 2018).

Before the secretion of mature toxin, the precursor protein pro-CyaA, unable to translocate across the plasma membrane of target cells, undergoes acetylation of AR by dedicated acyltransferase. This acetylation converts the inactive precursor protein to the active CyaA toxin which is then secreted via type I secretion system across the bacterial envelope. (Barry et al., 1991; Fiser et al., 2007; Glaser, Sakamoto, et al., 1988; Guermonprez et al., 2001) After secretion, it is proposed that the increased calcium levels induce the final conformational transition of the ACT (Cannella et al., 2017).

ACT has a central role in the early pathogenesis, further in colonization of *B. pertussis* in respiratory track (Goodwin & Weiss, 1990). To be able to transmit its effects, first ACT attaches to the cell surface CD11b/CD18 integrins through the C-terminal RTX region and inserting the HR to the cell membrane causing the cation selective pore-forming and the calcium influx to the target cell (Fiser et al., 2007; Guermonprez et al., 2001; Vojtova-Vodolanova et al., 2009). This influx promotes the TR assisted translocation of ACD and causes the haemolytic effects (Fiser et al., 2007). After translocation to the target cell cytosol the N-terminal catalytic, calmodulin-activated ACD is responsible for extremely rapid conversion of signalling molecule cAMP from ATP and disruption of the physiology of the cells in respiratory track, thus inhibiting the oxidative burst and complement-mediated opsonophagocytic killing of bacteria and allowing the colonization of *B. pertussis* (Ostolaza et al., 2017; Vojtova et al., 2006). Moreover, ACT has been found to have inhibitory effects on biofilm formation, but its significance in *B. pertussis* pathogenesis remains unclear (Hoffman et al., 2017).

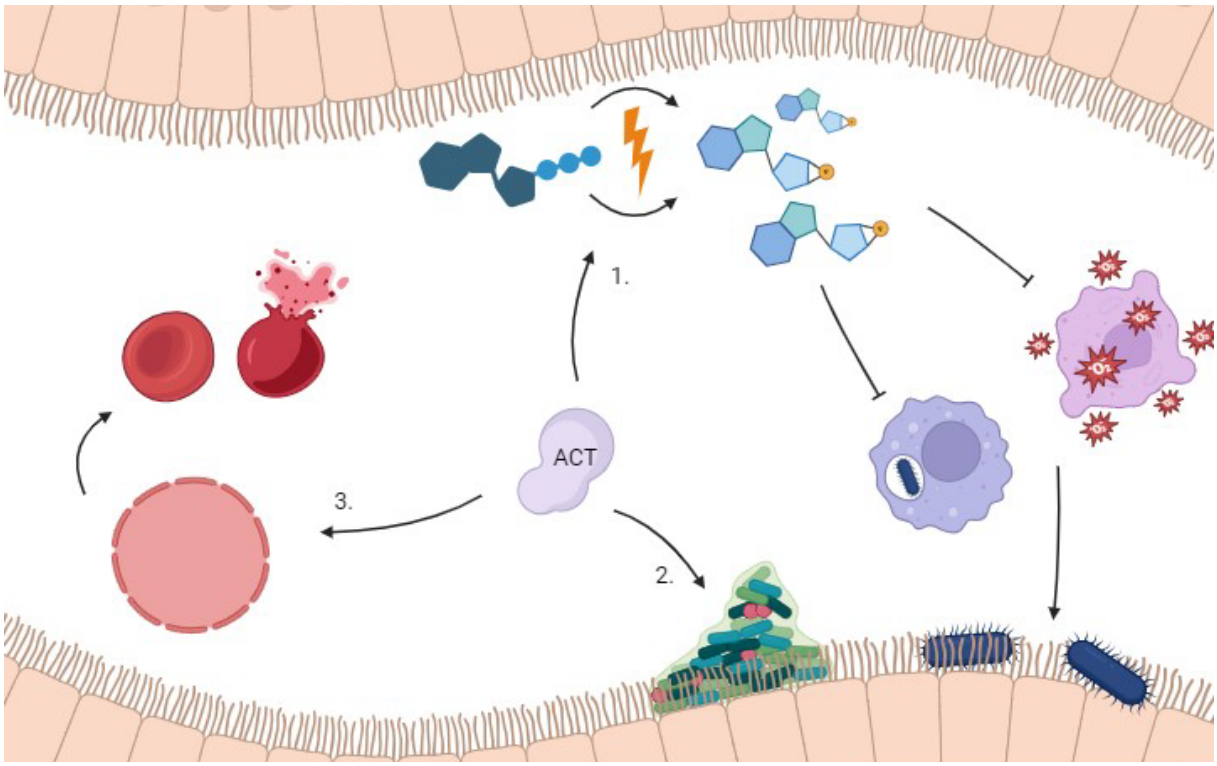

Figure 7. Contribution of adenylate cyclase toxin to *B. pertussis* pathogenesis. ACT is known to 1.) Cause extremely rapid conversion of cAMP from ATP and disrupt of the physiology of the cells in respiratory track thus, inhibiting the oxidative burst and complement-mediated opsonophagocytic killing of bacteria. 2.) Have inhibitory effects on biofilm formation. 3.) Cause haemolytic effects of *B. pertussis*. This figure is created by Vili Niinikoski with Biorender.com.

1.2.2 Immune evasion strategies of *B. pertussis*

Bacterial lineages evolve constantly and early changes in *B. pertussis* were seen in wP vaccine era, suggesting that vaccination was one of the major force driving changes in *B. pertussis* populations (Advani et al., 2013; Bart et al., 2014; Xu et al., 2015). Vaccines containing certain strains with certain pertussis toxin S1 subunit (ptxA), PRN alleles, pertussis toxin promoter (ptxP) allele and serotype were thought to lead the *B. pertussis* to adapt in vaccine induced immunity (Bart et al., 2014; Bouchez et al., 2021; Parkhill et al., 2003). As early as in 1970s ptxA1 allele become predominant in Finland due to wP-vaccines not containing the ptxA1 variant. The second observable shift occurred between 1980 and the 1990s, as the dominant pertactin allele transitioned from prn1 to prn2. Additionally, fimbrial serotypes Fim2, Fim2.3, and Fim3 have consistently reported in the *B. pertussis* population over the years, with variations observed solely in the prevalence of these serotypes. (Elomaa et al., 2005) To be

noted, *B. pertussis* is considered as an extremely homogenous bacterium (Sealey et al., 2015). However, after the first signs in wP era, the effect of aP vaccines on the adaptation of *B. pertussis* on vaccine induced immunity cannot be ignored. After the introduction of aP vaccines in 1990s the selective pressure against *B. pertussis* virulence factors changed from wP vaccines including thousands of antigens to the current 1 to 5 antigens of aP vaccines (J. L. Liang et al., 2018). This narrowed selection pressure has reportedly thought to led to the alterations of virulence factors and the expression of them. This has mostly happened into the virulence factors known as vaccine components but also to the other virulence factors of *B. pertussis*. These alterations include deficiencies of vaccine components, changes in FIM serotype distribution, point mutation ptxP allele and differentiated antigen expression levels. (Barkoff et al., 2019; Heikkinen et al., 2008; Martin et al., 2015; Mooi et al., 2009; Zomer et al., 2018) Strong evidence behind this vaccine-driven selection has been established and associated with the resurgence of pertussis (Barkoff et al., 2019).

1.2.2.1 Vaccine antigen deficiencies of circulating B. pertussis

Deficiencies, meaning total shutdown of the expression in PRN have been previously reported (Barkoff et al., 2012, 2019; Bouchez et al., 2009; Weigand et al., 2018). Notably in Finland, no other defects in *B. pertussis* antigen production than pertactin deficiency have been previously reported (Barkoff et al., 2014). After introduction of aP-vaccines, the first PRN and PT deficient isolates were reported in France in 2007 (Bouchez et al., 2009). In Finland, first PRN deficient isolates were reported 2 years after introduction aP vaccine containing PRN (Barkoff et al., 2012). Later on, isolates not producing PRN reach high proportions of circulating isolates, whereas deficiencies in PT, FHA and FIM have remained as few in Europe (Barkoff et al., 2019). According to Eurosurveillance study with panels of isolates from 11 European countries within 2012-2015 PRN deficient isolates constituted up to 25% (66/265) of tested isolates. In the same study the relation between time aPs used and increasing prevalence of PRN deficient isolates were established. (Barkoff et al., 2019) In addition, isolates with multiple deficiencies have been reported (Bouchez et al., 2015).

1.2.2.2 PtxP point mutation

One factor behind the enhanced fitness against vaccine induced immunity and resurgence of pertussis is reported to be a point mutation in the promoter region of pertussis toxin. The mutation possibly enhances the binding of the *B. pertussis* virulence gene (*Bvg*) A, the global regulator of virulence gene expression in *B. pertussis*. This "novel" allele ptxP3 is associated with increased PT expression superior to the ptxP1 allele. (Gates et al., 2017) Several factors support the issue that the ptxP3 prevalence increased due to vaccine induced selection pressure. Firstly, PtxP3 allele was first time reported in the 1990s after the introduction of the wP vaccines becoming dominant after introduction of aP vaccines in the countries that has implemented it. Secondly, no ptxP3 alleles were found in pre-vaccine era and ptxP3 allele incidence was lower in countries which started vaccination later. All in all, the replacement of ptxP1 by ptxP3 is observed in many countries making it a global phenomenon. (Mooi et al., 2009)

The association between ptxP3 and the resurgence of pertussis was reported in Finland during the epidemic in 2003 (Elomaa et al., 2007). In addition, PT has been associated with severity of the disease and the epidemiological shift of infecting older people (Mooi et al., 2009; K. Scanlon et al., 2019).

1.2.2.3 Alterations in the expression level of non-vaccine component virulence factors

In addition to the increased expression of PT due to the point mutation in the promoter region, other alterations in the expression of *B. pertussis* virulence factors have also been reported. These previously reported alterations occurred in the virulence factors under a two-component regulatory locus, the *bvg*, or *vir*, locus, accountable for regulating the expression of majority of the virulence factors (Cummings et al., 2006; Gates et al., 2017; Stibitz et al., 1989). Increase in the expression of two complement regulatory proteins, autotransporter virulence-associated gene 8 and *B. pertussis* resistance to killing antigen (BrkA) of *B. pertussis* have been associated with the improved evasion of complement mediated killing and the fitness of *B. pertussis* in the host. However, only a small number of strains in these studies were included. (Brookes et al., 2018; Stefanelli et al., 2006)

Expression of other virulence factors under a regulation of *bvg* or *vir* locus, such as FHA, PRN, and PT has remained less studied (Moon et al., 2017). However, in this thesis we provide

exclusive information of the alterations happened in the expression of PRN, PT, FHA and ACT in Finnish clinical isolates during 1991-2020.

2 Aims and hypotheses

With all these previously reported immune evasion strategies of *B. pertussis* in mind, we hypothesize that alterations in the expression of the virulence factors of *B. pertussis* for example to compensate the pertactin deficiency could be discovered. We also hypothesized that these alterations could be timely associated with the introduction of aP vaccines to strengthen the statement that aP vaccines has affected the development of the *B. pertussis*.

The primary aim of this study was to develop a sensitive and specific assay to examine whether the expression of four virulence factors mentioned previously of *B. pertussis* has altered within the study period.

Secondary aim was to find the timely association between found alterations and the aP vaccine introduction to prove the concept that aP vaccines are driving the *B. pertussis* to evolve in certain direction to survive in aP vaccine induced immunity.

We hypothesized that these possible alterations happened in the *B. pertussis* virulence factor expression could result as one factor behind the lessened vaccine efficacy and resurgence of the pertussis that has happened after the introduction of the aP vaccines.

3 Results

3.1 Validation of the virulence factor expression measurement assay

3.1.1 Assay specificity, variability, and accuracy

The assay showed adequate specificity and was able to reach same expression status of the isolates than previously tested.

Coefficient of variation (CV) was employed to describe the assay variability and precision. CV serves as a widely accepted metric for summarizing the variability in continuous-type chemical assays such as Enzyme-linked immunosorbent assay (ELISA) and other similar methods. Our assays precision underwent evaluation at two levels: inter-assay, which involved examining the consistency of replicates within the same test, and intra-assay, which involved assessing the precision across independent tests.

For inter-assay, CV percentages were calculated for each virulence factor, while intra-assay CV percentages were calculated for each isolate and for each virulence factor by using the mean value of each individual test. Intra-assay CV ranges for each antigen in table 1.

The assay developed, showed an adequate precision, with inter and intra- assay coefficients of variation lower than 10% for all virulence factors measured.

Table 1 Co-efficient of variation in inter and intra-assays.

	PRN	PT	FHA	ACT
Inter-assay, CV-%	4,0%	5,15%	4,94%	4,86%
Intra-assay, CV-% (range)	3,3-7,1%	4,6-6,2%	3,3-5,8%	3,0-5,7%

In addition to show accuracy of our assay, we interpreted the standard curves of our assay for each of measured virulence factor. (Figure 7). Measured, non-normalized expression level (absorbance) of each isolate and virulence factor in this thesis fell into the linear range interpreted (see table 2.) proving that within interpreted linear ranges our assay is accurate and able to measure possible changes in virulence factor expression.

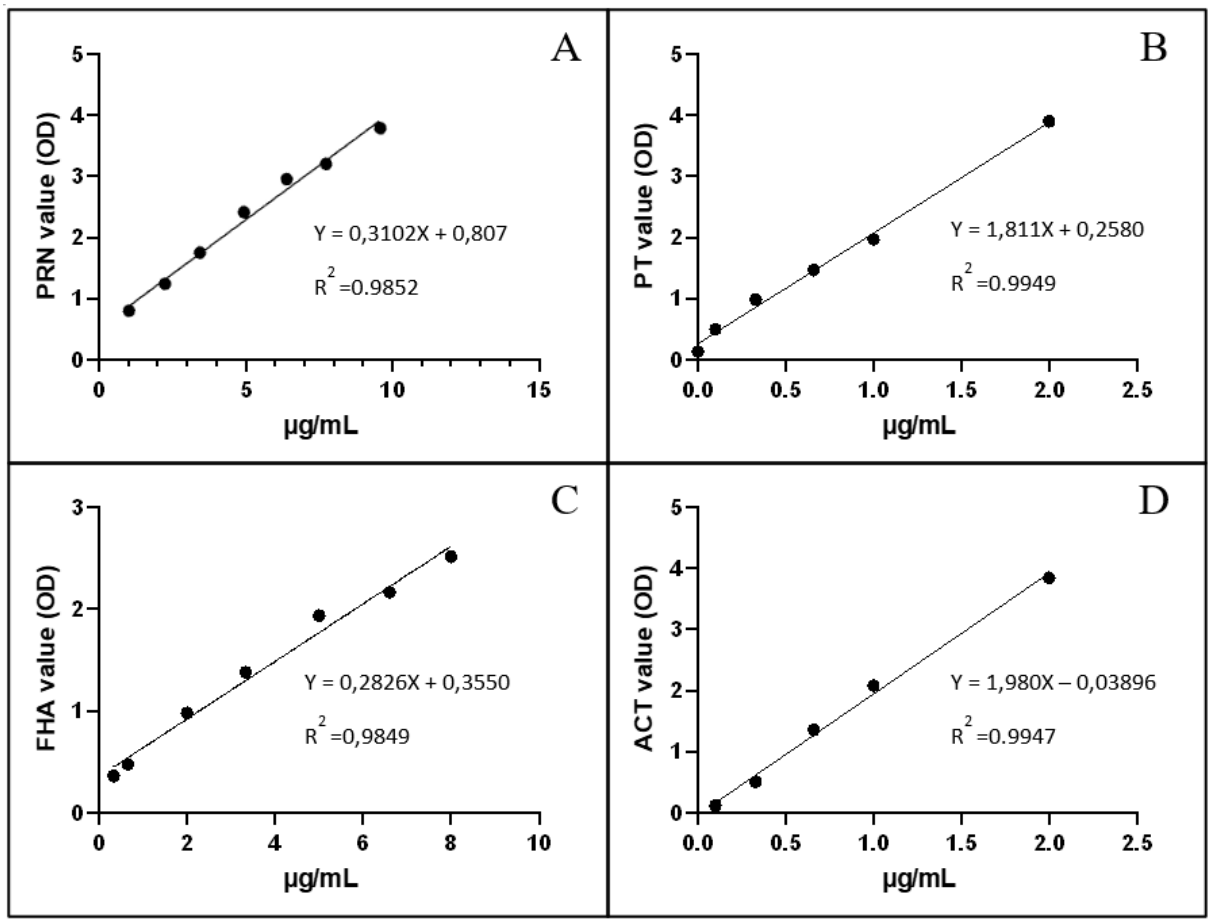

Figure 8. Standard curves of virulence factor expression ELISA. Interpreted standard curves for expression-ELISA-assay. Standard curves were interpreted for all virulence factors measured: PRN (A), PT (B), FHA (C) and ACT (D). OD = Optical density.

Table 2. Assay linear ranges and measured values

	PRN	**PT**	**FHA**	**ACT**
Linear Range of the assay (Absorbance)	0,81 – 3,9 (R2= 0,9852)	0,26 – 3,88 (R2= 0,9949)	0,36 – 2,62 (R2= 0,9849)	0,10 – 3,92 (R2= 0,9947)
Range of measured values (Absorbance)	0,83 – 3,56	0,34 – 1,37	0,55 – 2,15	0,46 – 2,86

3.2 Virulence factor expression

3.2.1 Pertactin expression

In this thesis, out of tested isolates from 1991 to 2020, 46 (15%) comprised PRN deficiency. First Finnish PRN deficient isolates was isolated in 2011. Within the study period increasing

prevalence of pertactin deficient isolates was recorded. Yearly prevalence of tested PRN deficient isolates since 2011 in Finland shown in Table 3. In addition to the increased proportion of PRN deficient isolates, the expression level of PRN fluctuated between 0,41 AU to 1,87 AU and the significant decrease in PRN expression level within study period was recorded (<0,05, Kruskal-Wallis).

Table 3. Prevalence of pertactin deficient *B. pertussis* isolates in Finland

Proportion of pertactin deficient isolates within isolates isolated by Finnish Reference Laboratory for Pertussis and Diphtheria

	2011	**2012**	**2013**	**2014**	**2015**	**2016**	**2017**	**2018**	**2019**	**2020**
amount	2/10	1/7	1/4	1/7	2/5	4/10	8/11	10/21	13/18	5/17
%	20%	14%	25%	14%	40%	40%	72%	48%	72%	29%

3.2.2 Pertussis toxin expression

The expression level of pertussis toxin fluctuated between 0,39 AU to 2,40 AU within the tested isolates. Among of the Finnish isolates studied in the period 1991-2020 none were found to be PT deficient. However, alterations in expression of PT were revealed. The results show that the expression of PT increased significantly (<0,05, Kruskal-Wallis) within study period. In detail, PT expression exhibited an upward trend until the period of 2001-2005, coinciding with the introduction of aP vaccines. 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).

3.2.3 Filamentous hemagglutinin expression

The major alteration was found in the increase of expression level of FHA resulting in 2,1-fold increase within the study period of 1991-2020 (<0,001, Kruskal-Wallis). The accelerated 1,75-fold increase of FHA resulted increase after the period of 2006-2010 (<0,001 Mann-Whitney U test). The expression level of FHA fluctuated between 0,86 AU to 5,71 AU within the tested

isolates. None of the 302 isolates studied in the period 1991-2020 were found to be FHA deficient.

3.2.4 Adenylate cyclase toxin expression and adenylate cyclase activity

Within the study collection, the statistically significant 1,7-fold increase (<0,01, Kruskal-Wallis) in expression of ACT within the study period was observed. An accelerated 1,5-fold increase (<0,01 Mann-Whitney U -test) in ACT expression, timely simultaneously with accelerated increase of expression in FHA was observed after 2006-2010. The expression level of ACT fluctuated between 0,49 AU to 4,52 AU within the tested isolates. After examination of the isolates, ACT deficient isolate remains undiscovered. Ac activity test performed parallel to ACT expression ELISA revealed over 1,8-fold increase between activity of randomly selected isolates isolated before 2008 and isolates isolated after 2008 (data not shown). Strong correlation between isolates AC activity and ACT expression was discovered (r=0,74, r^2=0,55).

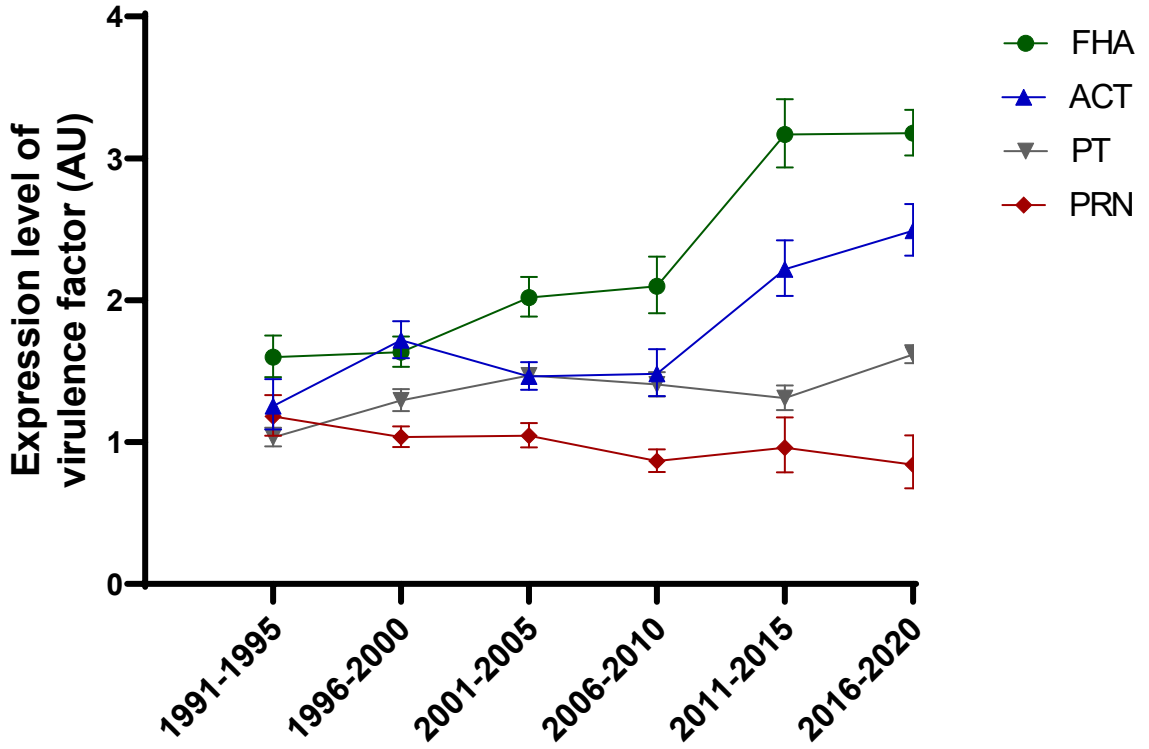

Figure 9. Alterations in virulence factor expression level of *B. pertussis* in 1991 to 2020. Median expression levels of Finnish *B. pertussis* isolates virulence factors, FHA, ACT, PT and PRN in arbitraty units (AU) of each period. Number of tested isolates in the periods 1991-1995; 35, 1996-2000; 60, 2001-2005; 61, 2006-2010; 34, 2011-2015;35, and 2016-2020; 77. 95% confidence intervals presented.

3.3 Alterations in expression of virulence factors in isolates with different fimbrial serotypes and ptxP alleles

After examination of expression levels of virulence factors, the virulence factor expressions of isolates with different FIM serotypes and ptxP-alleles were compared. This comparison revealed significant difference in the expression of PRN and PT mean values between isolates with FIM2 (n=162) and FIM3 (n=106) serotypes. Significantly higher expression of PRN was observed in isolates with FIM2 (1,08 AU) serotype compared to isolates with FIM3 serotype (0,88 AU) ($p<0,0001$, Mann-Whitney U -test). Furthermore, significantly lower expression of PT was also associated with isolates with FIM2 serotype (1,38 AU) rather than with isolates with FIM3 serotype (1,49AU) ($p<0,001$, Mann-Whitney U -test). No significant difference in expression of FHA or ACT was found between Fim2 and Fim3 carrying isolates.

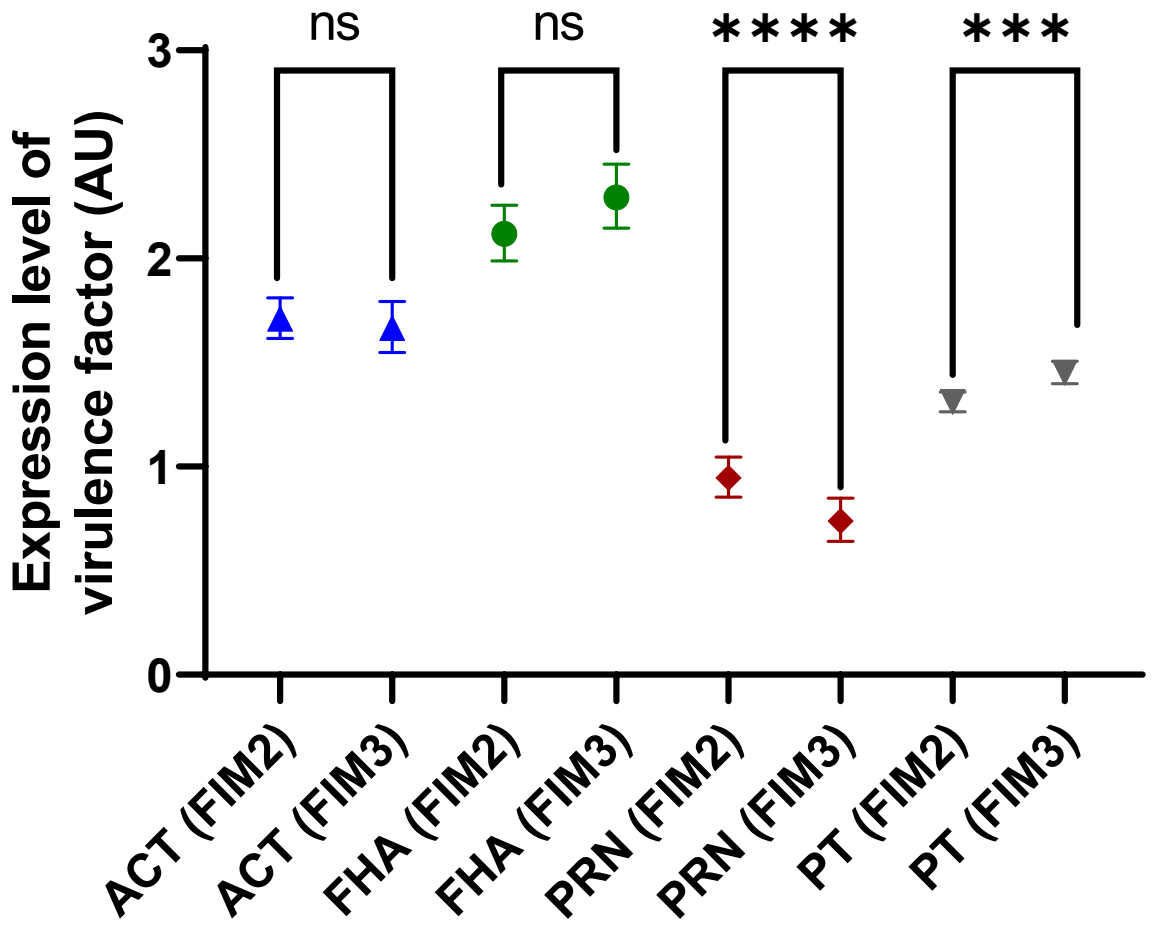

Figure 10. Alterations between FIM2 and FIM3 serotype isolates in expression of virulence factors. Median values (arbitraty units) of each virulence factor compared between FIM2 and FIM3 isolates. 95% confidence intervals presented. The statistical tests were performed with Mann-Whitbey U test, ns. > 0,05, *** < 0,001, **** < 0,0001.

The same comparison was performed also between isolates with ptxP-alleles ptxP1 and ptxP3, revealing significant differences in expression of all studied four virulence factors. Out of these, significantly higher expression of ACT ($p<0,01$, Mann-Whitney), FHA ($p<0,001$, Mann-Whitney), and PT ($p<0,001$, Mann-Whitney) was observed in isolates with ptxP3 allele (n=155) compared to isolates with ptxP1 allele (n=85). In addition, PRN expression in isolates with ptxP3 allele was significantly lower than in isolates with ptxP1 allele ($p<0,001$). Mean values for expression (AU) of virulence factors of isolates with ptxP1 and ptxP3 alleles shown in Table 4.

Table 4. Virulence factor expression (AU) between ptxP1 and ptxP3 carrying *B. pertussis* isolates.

	ACT	PT	FHA	ACT
ptxP1	1,63	1,87	1,15	1,3
ptxP3	1,49	2,76	0,91	1,49

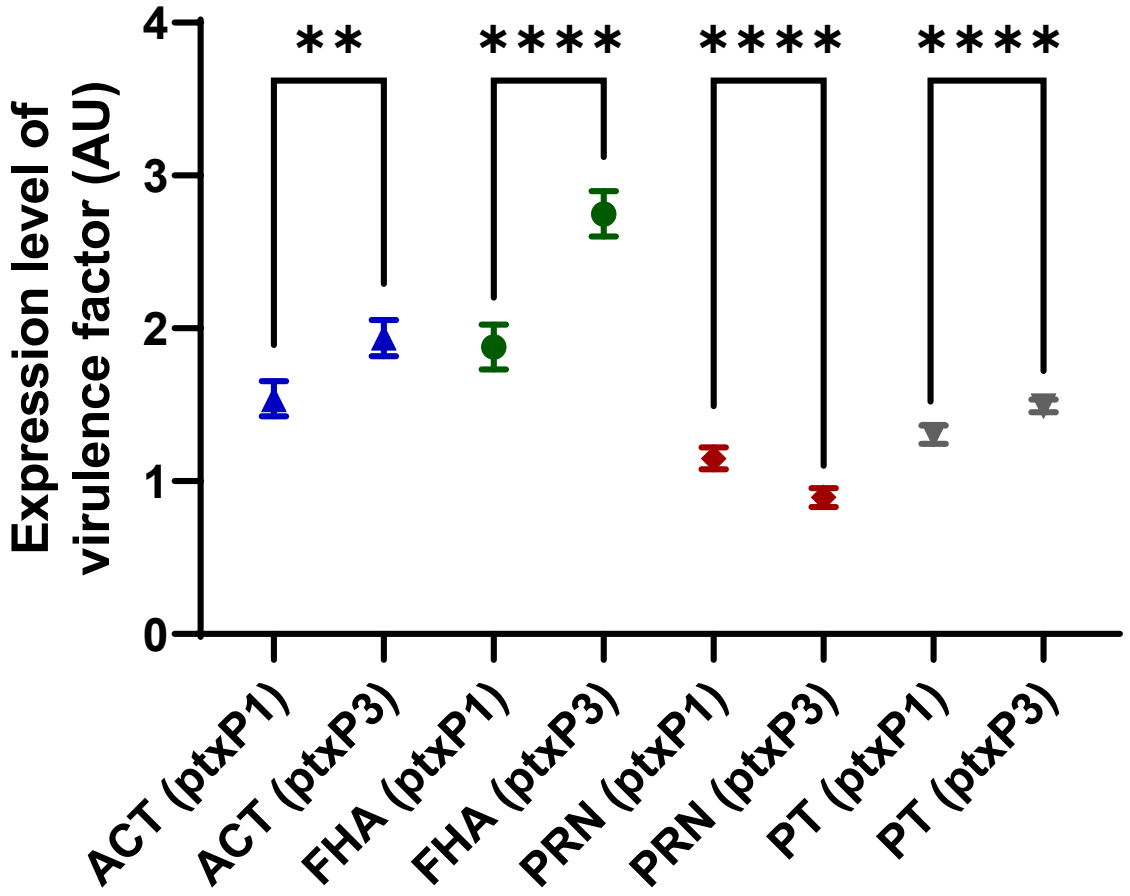

Figure 11. Alterations between ptxP1 and ptxP3 serotype isolates in expression of virulence factors. Median values (arbitraty units) of each virulence factor compared between FIM2 and FIM3 isolates. 95% confidence intervals presented. The statistical tests were performed with Mann-Whitney U test, ns. > 0,05, *** < 0,001, **** < 0,0001.

3.4 Correlation between filamentous hemagglutinin and adenylate cyclase toxin expression

While examining the values of virulence factor expression and possible correlations between different virulence factors, strong correlation (r=0,776) between FHA and ACT expression was

found in isolates collected after 2005. Correlation between FHA and ACT in isolates collected before 2005 was r=0,018. Correlations between other virulence factors were not found.

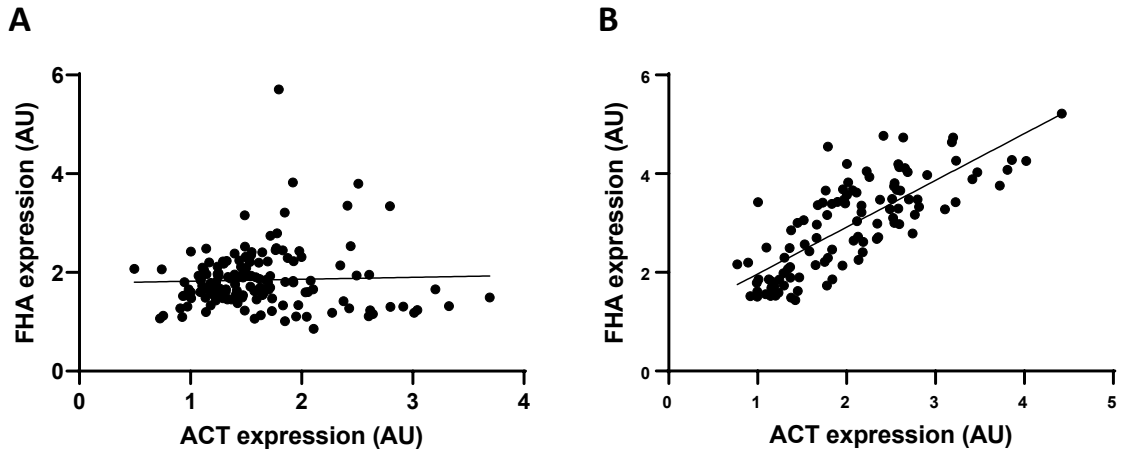

Figure 12. Correlation between expression of FHA and ACT before and after introduction of acellular pertussis vaccine. A) correlation between expression of FHA and ACT in isolates collected before 2005 (r2<0,01, r=0,036 Spearman correlation). B) correlation between expression of FHA and ACT in isolates collected a after 2005 (r2=0,598, r=0,784 p<0,01 Spearman correlation).

4 Discussion

Alterations within circulating isolates took place mainly after the introduction of aP vaccines and the main causative behind these alterations partly lies on aP vaccines (Bart et al., 2014). These reported alterations occurred rather fast after the implementation of aP vaccines, implying of the ability of *B. pertussis* to adapt quickly to changing circumstances. Good example of this is isolates with virulence factor deficiency. PRN deficiency is associated with the time since the introduction of aP vaccines (Barkoff et al., 2019; Martin et al., 2015). The first pertactin deficient isolates were reported in France 5 years after the introduction of aP vaccine including vaccine component PRN (Bouchez et al., 2009). In Finland, same happened 2 years after the implementation of three component vaccine including PRN (Barkoff et al., 2012). In both countries increasing prevalence of PRN deficient isolates are reported implying that lack of pertactin increases the survival rate of the bacteria in the aP vaccine (including PRN) induced immunity. In the other hand, low number of deficiencies in other virulence factors may suggest that lack of other virulence factors has not been beneficial to the *B. pertussis*. (Barkoff et al., 2019) In addition to deficiencies, also prevalence of isolates with increased expression of certain virulence factors has increased after introduction of aP vaccines (Brookes et al., 2018; Stefanelli et al., 2006). This again suggests that there could be alterations in other virulence factor expression to be found to understand better the ability of *B. pertussis* to evade immune system in the host.

Within this thesis, we aimed to develop a sensitive and specific assay to measure expression of four virulence factors of *B. pertussis* quantitatively and timely associate possible findings with the introduction of aP vaccines. The developed assay was proven to be consistent and precise with acceptable CV values. With this ELISA-based assay, we were able to discover the alterations in the expression of four antigens studied and strengthen the concept that aP vaccines with certain vaccine component composition drive *B. pertussis* to evolve in certain direction to be able to survive and evade in aP vaccine induced immunity.

While there are multiple previously reported alterations in virulence factor expression of *B. pertussis* associated with the introduction of aP vaccines, these recent findings introduced in this thesis, support even more of the hypothesis *B. pertussis* is able to adapt rather fast to the changing selection pressure. The results gained from this study showed significantly increased expression of three virulence factors. FHA expression doubled within the study period and the main increase was seen after the introduction of aP vaccines. Like FHA, ACT increased 1,5-

fold, and had the main increase after introduction of the aP vaccines. Also, PT expression increased within the study period, however this increase was not, at least, timely associated with the aP vaccine introduction. Although, this could be a result from increasing prevalence of isolates carrying ptxP3 allele which has been repeatedly associated with increased PT expression. However, the decrease in the expression of PT was reported after introduction of aP vaccines. This decrease would be effect of the adaptation of *B. pertussis* to the aP vaccines. Different from others, PRN expression remained stable and had to trend towards declining, even when PRN deficient isolates were excluded. In addition, the strong correlation between ACT and FHA expression were seen, but only after the introduction of aP vaccines. Also, increased expressions of all studied virulence factors were associated with ptxP3 allele rather than ptxP1 and significant differences in PRN and PT expressions were seen between FIM2 and FIM3 serotypes.

4.1 Unraveling reported alterations: Importance of Virulence Factors, Impacts, Alternatives, and the Interplay between FHA and ACT

4.1.1 Pertactin

PRN has been reported to cause strong immune response in humans (Pereira et al., 2010; Steinhoff et al., 1995). Therefore, it is considered as an ideal virulence factor to be used in vaccines against pertussis. However, this has led to the situation where the PRN deficiency is more beneficial to the *B. pertussis* than expressing it would be. After the introduction of aP vaccines and reports of first PRN deficient isolates, prevalence of PRN deficiency in *B. pertussis* isolates has steadily increased with a passage of time (Barkoff et al., 2019). As mentioned before the occurring of the isolates lacking the expression of PRN has heavily associated with the usage of the aP vaccines and more precisely aP vaccines containing PRN component (Barkoff et al., 2019).

While PRN deficient isolates have enriched, it has remained unknown whether this loss of a virulence factor expression is compensated somehow. The functions and importance of PRN have been debatable. Mostly known as adhesion protein of *B. pertussis* it has also claimed to have a role in bacterial shedding and immune evasion in studies performed with either *B. pertussis* or *B. bronchiseptica*. (Inatsuka et al., 2010; Ma et al., 2021; Nicholson et al., 2009; B. M. van den Berg et al., 1999)

The importance of PRN in adhesion has been questioned and the increase in isolates not producing PRN has proven PRN not to be essential for the *B. pertussis*. Additionally, increasing prevalence of circulating PRN deficient isolates implies that the lack of it may even increases the fitness and improves the survival of *B. pertussis* in highly aP immunized human populations. Whether the lack of PRN affects remarkably to the transmission of the disease, remains to be studied. However, this does not seem to be the case, while number of PRN deficient strains are increasing.

To study potential compensate for the loss of PRN, we studied one of the major adhesion protein, FHA, expression. Although we showed significant increase in expression of FHA, the increase was not associated only with the isolates with neither lower expression of PRN nor PRN deficiency. This indicates that if there is compensation happening, it might not be direct change between PRN and FHA but might be a sum of many other virulence factor expressions, facilitating adhesion like FIM, BrkA, dermonecrotic toxin or tracheal cytotoxin. Other possibility is that the importance of PRN in adherence is minor or the lack of it is not affecting to the adherence of *B. pertussis* on such large scale as pointed out by (B. M. van den Berg et al., 1999). However, these possible compensating factors to facilitate the PRN deficiency has been reported, including previous studies on BrkA increase by Stefanelli et al., 2006 and now FHA increase.

Associated with the bacterial shedding, PRN enhances and contagiousness of the bacteria by increasing the expulsion of the bacteria out of the body. This has been showed in mice populations, when two populations of mice are exposed to mouse infected with PRN (+/-) *B. bronchiseptica*. As a result, infection rate of PRN expressing infection was higher compared to the PRN deficient indicating that there could be reduced contagiousness of PRN deficient isolates in human hosts also. (Ma et al., 2021) However, showing this on humans is hard and until this date real world evidence of this has not been reported. One opportunity to study the contagiousness would be the human challenge model used in University Hospital in England, Southampton. In this model humans are exposed to *B. pertussis* to test for example the efficacy of vaccines. (de Graaf et al., 2017) Though, reaching the line of this kind of importance to test the contagiousness of different strains on humans are not expected to happen in near future.

In some countries like Finland and Slovenia, after the change from the three-component vaccine also including PRN to the two-component vaccine including only FHA and PT, the first sights of reversion of isolates expressing PRN have been seen (unpublished data). Altogether, this

indicates that even if the expression of PRN is compromised under certain circumstances, PRN is important to *B. pertussis* and the expression is restored whenever it is beneficial.

4.1.2 Pertussis toxin

PT is used as a vaccine component in all the aP vaccines (Esposito et al., 2019). Nonetheless only few PT deficient isolates have been reported worldwide (Barkoff & He, 2019). Simultaneously, increased expression of PT, shown also in this thesis, has been associated with the increase of ptxP3 allele as mentioned previously. This increased expression of PT may also contribute to the more effective immune evasion and colonization of the bacteria but also enhance the shedding of the bacteria by strengthening the characteristic symptoms. (Mooi et al., 2009)

Just the fact that PT is *B. pertussis* specific indicates that it has significant role in the pathogenesis of the bacteria. The importance of PT has proven out based on the only few PT deficient isolates reported previously (Barkoff & He, 2019). However, deficiency in PT does not render the bacteria completely avirulent but the absence or reduced production of PT can significantly influence the bacterium's ability to establish infection, evade immune responses, and cause severe pertussis symptoms (K. Scanlon et al., 2019).

4.1.3 Filamentous Hemagglutinin

FHA primarily known for its ability to promote bacterial attachment and adherence to host cells in the respiratory tract (E. I. Tuomanen & Hendley, 1983). FHA mediates adherence by binding to a variety of host cell receptors, including ciliated cells, neutrophils macrophages, and epithelial cells (Smith et al., 2001; E. Tuomanen & Weiss, 1985). This attachment facilitates bacterial colonization of the respiratory tract and causing infection. Besides FIM, FHA is reported to be crucial in adherence of *B. pertussis* on ciliated cells. (E. I. Tuomanen & Hendley, 1983)

Despite of the strong immunogenicity and wide usage of FHA as vaccine component similar prevalence of FHA deficiency than PRN has not been reported. Previously, there is only few FHA negative isolates found reportedly, which indicates of the importance of this virulence

factor for *B. pertussis*. (Bart et al., 2015; Hegerle et al., 2012; Weigand et al., 2018) In addition, the surge in the expression indicates of the increased role of FHA in the aP induced immunity. Due to many essential functions of FHA, benefits of increase of it are not restricted to one function but are most likely resulting also in enhanced adhesion and immune evasion of *B. pertussis*.

Even if strong immune response against FHA is achieved through aP vaccines including FHA, the expression of this virulence factor is not reduced or terminated. Due to its importance for *B. pertussis*, the expression of FHA is increased to hypothetically enhance the survival of the bacteria by inhibiting immune defence, enhancing colonization due to more effective adhesion, and hedging from immune defence rather than terminated to hide from aP induced immune defence. This leads to a battle between strong immune response against FHA and the inhibition of immune defence by *B. pertussis*.

4.1.4 Adenylate cyclase toxin

As mentioned previously ACT is an important virulence factor assisting colonization and immune evasion of *B. pertussis* by interfering the normal function of immune system (Ostolaza et al., 2017; Vojtova et al., 2006). It is the only virulence factor studied in this thesis which has not been used as component of aP vaccines, and only the immunity induced by the natural infection has set the selection pressure for ACT (J. L. Liang et al., 2018). Due to this and the role of ACT in the pathogenesis of the *B. pertussis*, we expected that the aP induced narrowed selection pressure, could have led to the increased expression of ACT, for example to compensate reduced efficacy in the adhesion of *B. pertussis* due to the loss of PRN. However, no such important correlation between PRN deficiency or PRN expression and ACT were found.

One clear sign of the importance of ACT is that not a single ACT deficient isolate has been reported (Barkoff & He, 2019). However, it is important to keep in mind that the selection pressure in aP vaccine era has not been targeting ACT as it has not been used as vaccine component (J. L. Liang et al., 2018). Nevertheless, the increase in ACT expression shown in this thesis suggests that the importance of ACT has increased in aP vaccine era, but the outcome of the increased ACT expression remains uncovered within this thesis. However, the main increase in the expression of ACT took place before the first PRN deficient isolates were

reported in Finland, so the increase of ACT and FHA may not be a consequence of decreased and terminated expression of PRN based on these results. Additionally, only speculations of that the increased ACT expression could result as enhanced colonization potency, inhibition of immune defence and with these to persistent infection can be drawn.

ACT clearly shows importance to *B. pertussis*. Its known functions indicates that the increase is enhancing the colonization and immune evasion of the bacteria but whether there are other outcomes of the increase of ACT remains to be discovered. After proving itself as important virulence factor with increased expression ACT could be considered as a good vaccine component for further vaccine development.

4.1.5 ACT and FHA interplay

ACT and FHA, both recognized as significant virulence factors in the development of pertussis, serve distinct roles in the colonization of the bacteria while also being interconnected (Hoffman et al., 2017; Perez Vidakovics et al., 2006). Unlike other Repeat-in-toxins that are secreted into the extracellular environment, ACT has ability to remain associated with the bacterial cell surface (Hewlett et al., 1976). Previous studies have demonstrated an association between ACT and FHA on the cell surface, as the absence of FHA has been linked to increased release of ACT to the extracellular milieu (Zaretzky et al., 2002). This interaction has shown to facilitates the functioning of both virulence factors (Perez Vidakovics et al., 2006). In a study conducted by Perez Vidakovics et al. in 2006 it was observed that mutants lacking either FHA or ACT exhibited a significantly reduced ability to adhere to host cells. Additionally, the adhesion function of ACT could not be explained by adhesin-like activity, suggesting that ACT promotes the functionality of FHA in facilitating adherence.

Vice versa, the interaction between ACT and FHA has been shown to increase the cAMP generation compared to the free ACT molecules. These results have raised the hypothesis that FHA functions as delivery mechanism for ACT to more robust intoxication of host cells. In addition, ACT is reported to regulate the biofilm formation mediated by FHA by decreasing the amount of biofilm formed, through interaction with FHA. (Hoffman et al., 2017)

As previous studies have shown the close interaction and cooperation between ACT and FHA it is unsurprising that the expression of these two virulence factors has increased side by side (Hoffman et al., 2017; Perez Vidakovics et al., 2006; Zaretzky et al., 2002). This interaction

and the cooperation also strengthen the importance of these both virulence factors even more due to their partly impaired efficacy when functioning alone. By missing one of these virulence factors, the *B. pertussis* suffers from lack of effectiveness in adhesion and immune to evasion which contributes directly to the colonization of the host. (Perez Vidakovics et al., 2006)

4.1.6 Possible Associations between reported alterations of *B. pertussis*

aP vaccines with limited components induce immune protection against certain virulence factors of *B. pertussis*. This has driven *B. pertussis* to adapt and change virulence factor expression to less immunoreactive direction. However, these changes do not happen without compromises. For example, in the case of PRN, which causes high immune response, the most beneficial alteration for *B. pertussis* was the shutdown of the expression. Other side of this change is that the deficiency in PRN expression may contribute different adherence and lower transmission of the bacteria. (Ma et al., 2021)

However, total shutdown of expression has not been seen increasingly with other vaccine components. This for example may indicate the irreplaceability of the role of FHA in adhesion and PT in suppressing host functions. Thus, what would be the explanation to the decreased importance of PRN and the increasing prevalence of PRN deficient isolates? In this thesis we showed already increased expression of FHA and ACT, before the first PRN deficient isolate in Finland was reported. The increased expression and correlation between the expression of ACT and FHA, shown in this thesis, suggest that the cooperation of these two virulence factors has been even more significant after the introduction of aP vaccines and this could result as more effective adhesion. Other previous studies have shown the increased expression of virulence factor, BrkA which contributes with the serum resistance and adherence of the bacteria to the host epithelial cells (Ewanowich et al., 1989; Fernandez & Weiss, 1994). In addition to these, changes in the FIM serotype have been also reported by (Heikkinen et al., 2008), which most likely have also been affecting the possibility of PRN expression shutdown. Altogether, major changes in the expression of virulence factor related with the adhesion of the bacteria has taken place before the PRN deficient isolates were reported in Finland. Whether these changes have preceded and facilitated the PRN deficiency of the isolates remains still to be discovered.

4.2 Vaccines Before, Now and in the Future

Vaccination has been crucial in controlling and preventing the spread of pertussis including both wP and aP vaccines. Out of these two the aP vaccine is more widely used in developed countries and in these days has almost substituted the use of wP vaccines (Kuchar et al., 2016). Both of the vaccine type has typical advantages and disadvantages.

The aP vaccines, with narrowed selection pressure against *B. pertussis,* has its limitations in the disease prevention which may have caused the resurgence of pertussis. Priming with aP vaccine predominantly elicits humoral responses which enables *B. pertussis* use aP primed individuals as reservoirs. (Brummelman et al., 2015; van der Lee et al., 2018; Warfel et al., 2014) In other hand, aP vaccines may provide shorter duration of protection compared to wP vaccines. Some studies suggest that immunity wanes over time, leading to an increased risk of pertussis in vaccinated individuals, especially during adolescence and adulthood. (Schwartz et al., 2016) This difference and the increased expression of immune protective virulence factors, explains the gradual shift in the in epidemiology of *B. pertussis* to infect more and more adolescents and adults and use them as reservoir (Esposito & Principi, 2016).

Contrasting with aP vaccines, following priming with wP vaccine elicits, longer and robust humoral and cellular immune responses, broader protection due to number of antigens, reduced severity and duration of the disease and immune evasion prevention of *B. pertussis* (Cole et al., 2020; Gambhir et al., 2015; Sheridan et al., 2014; van der Lee et al., 2018; Warfel et al., 2014). However, they come with certain drawbacks. wP vaccines are associated with high reactogenicity including more local and systemic side effects, including pain, fever, and, rarely, more serious reactions such as seizures (Cody et al., 1981). This has led to the safety concerns and reduced public acceptance. To maintain the high vaccine coverage, the public safety and high acceptance are the key factors.

Even if, problems such as early waning immunity, lessened vaccine efficacy and pleomorphic properties has been associated with aP vaccine era, the aP vaccines are highly efficacious and has remarkable safety profile compared to the wP vaccines (Blumberg et al., 1991; Cody et al., 1981). The aP vaccines offered a solution to the reduced acceptance and coverage of pertussis vaccination after wP vaccine era. In other hand, studies comparing aP and wP vaccine efficacy have shown better efficacy of wP vaccines (Fulton et al., 2016). Also, Individuals vaccinated only with aP vaccines have shown to have 1,6 times higher risk to get pertussis compared to wP vaccinated or a combine schedule vaccinated in group of school age children (Belchior et

al., 2020). There have been speculations to support the aP vaccines claiming that the resurgence of pertussis is due to better knowledge and increased diagnostics of pertussis (Mooi et al., 2014). However, increasing evidence also supports the hypothesis that current vaccinations have been at least partly involved with current situation (Bart et al., 2014).

As pertussis remains an increasing global health concern, particularly for vulnerable populations such as infants and young children, the future poses questions regarding the potential escalation of pertussis prevalence and the ability of current aP vaccines to maintain efficacy against the ever-evolving *B. pertussis* strains. At this point with current aP and wP vaccines we are balancing between safety and efficacy of the vaccinations, and we fall far short for optimal disease prevention. The future vaccine development should pursue to interventions cherishing the positive properties of both vaccines to develop safe vaccine with improved durability and efficacy of protection. Encouragingly, there are currently multiple ongoing efforts to develop new approaches and vaccines against pertussis. These include improved antigen selection, dosage, adjuvants, administration sites and maternal immunization. (Amirthalingam et al., 2016; Cheung et al., 2006; Dias et al., 2013; Keech et al., 2023; D. Li et al., 2022; Roberts et al., 2008)

One of the new approaches use outer membrane vesicles (OMV). OMVs are vesicles released from the outer membranes of Gram-negative bacteria. They contain immunogens expressed in the outer membrane of bacteria which allows the immune response to preferentially target surface-exposed epitopes in native conformation. The benefits that OMV based acellular vaccination offers, include for example: Wide scale of immunogens located in *B. pertussis* membrane, possibly afford protection against antigen cooperation and protection against immune evasion due to wide scale immunogens. In case of resurgence of pertussis OMVs possesses right factors to prevent the current problem with aP vaccines including only few antigens. However, pertussis OMV vaccine has only been tested with animals and the safety and efficacy profile on humans still needs to be examined. (Roberts et al., 2008)

Other new approach is the BPZE1, an intranasal live attenuated pertussis vaccine. Knowing the fact that *B. pertussis* a strictly mucosal respiratory pathogen that primarily infects the upper airways the primary defence at the site of infection gained from BPZE1 would be beneficial against pertussis. This totally differs from current intramuscular route of administration of pertussis vaccines. Currently, BPZE1 is in the clinical trials in the phase 2b. According to this phase 2b trial, BPZE1 induces broad pertussis-specific mucosal and systemic immune

responses and might also prevent infection and transmission of pertussis. It is well tolerated due to its intranasal administration and showed acceptable safety profile. (Keech et al., 2023)

Whether these new approaches are enough to control pertussis more efficiently remains to be discovered in the future. However, to restrain the constantly evolving bacteria by maintaining the effective prevention of the disease it is essential to strengthen surveillance systems to monitor circulating pertussis strains and their genetic variations. This information can guide vaccine development efforts and ensure the up-to-date information of circulation strains to find right approaches to prevent pertussis in future and develop robust vaccines against pertussis.

4.3 Strengths and limitations of the study

In Finland, where vaccination coverage against pertussis is high, 89%, in 2021 and only one vaccine including certain components has been used at the time, possible effects of these vaccines can be examined more feasibly. (OECD, (accessed, 01 February 2024).; Versteegen et al., 2021) Also, isolated geographical location decreases the bacterial strain crossing with foreign ones and possibly enables the preservation of endemic strains better when compared to the central European countries. However, it's important to note that the data used in the study only includes information on isolates circulating in Finland. This limitation restricts the generalizability of the study's findings to the entire world until further investigations are conducted to examine expression alterations in other countries.

To address this problem, we developed a feasible, precise, and consistent assay for directly measuring the expression of these four virulence factors of *B. pertussis* that is usable for larger scale studies. At this point, the assay is optimized only for these four antigens, but with some effort, large scale studies of expression of other virulence factors of *B. pertussis* could be performed in the future. This might point out useful considering the upcoming vaccine development. Furthermore, to conduct a comprehensive study across Europe to gain a deeper understanding of the effects of the aP vaccines on the alterations of *B. pertussis*, we have collected isolate panels from every participating country as part of the EUpert-study. This collection of isolates will allow us to examine the expression of virulence factors in other European countries in the future, thereby providing valuable insights into the broader implications of the aP vaccines.

As an inevitable outcome of conducting such an extensive study, this investigation was carried out in a laboratory setting. This might affect in some manner to the expression levels of the isolates due to the processing of the clinical samples collected. Nonetheless, the study gives wide insight of the alterations in the expression of these four virulence factors associated with aP vaccines, never reported before. The inclusion of a large number of randomly selected and tested samples enhances the robustness of the findings in this study. Additionally, considerable amount of previously collected data on ptxP alleles, FIM serotypes, and certain patient information significantly enhanced the depth of understanding in the alterations of *B. pertussis*.

5 Materials and methods

5.1 Bacterial strain selection and cultures

Within the study period from 1991 to 2020, 904 clinical isolates of *B. pertussis* were isolated in Finland by Pertussis Reference Laboratory of the National Public Health Institute, 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. The isolates were grouped timely on 5-year time periods shown in Table 5. Study set comprised out of 156 isolates collected before introduction of aP vaccines (1991-2004) and 146 isolates collected after the introduction of aP vaccines (2005-2020).

Table 5 Number of tested *B. pertussis* isolates in each 5-year time period

Time period	1991-1995	1996-2000	2001-2005	2006-2010	2011-2015	2016-2020
Number of isolates tested	35	60	61	34	35	77

The randomly selected isolates were cultured on Bordet-Gengou medium containing 15% sheep blood in 35°C for 72 hours. In addition, isolates were further cultured in 150µl Stainer-Scholte medium on Microlon® high-binding microtiter plates (Greiner Bio-One GmbH, Frickenhausen, Germany) in shaker 35°C, 150rpm for another 24h. The isolates were inoculated to Stainer-Scholte medium to reach bacterial density of 0,05 +/- 0,01. After 24h culture bacterial density reached from 0,1 to 0,25 accordingly. The Bacterial densities measured with plate reader (Victor Nivo^{TM} multimode, Perkin Elmer, Turku, Finland) 600nm.

5.2 Development and validation of quantitative ELISA-based assay for measuring virulence factor expression levels

Previously there has not been a method for direct quantitative measurement of *B. pertussis* virulence factor expression. The methods used previously have been consisted of semi-quantitative and other non-direct measurement not able to measure the direct amount of virulence factors. (Barkoff et al., 2012, 2014; Heikkinen et al., 2008; Tsang et al., 2005) To study the alterations in the expression of *B. pertussis* virulence factors, optimized, and validated

the assay to measure expression of four virulence factors, ACT, FHA, PRN and PT precisely and accurately were developed. This protocol is validated only for four virulence factors mentioned above. However, with further validation this assay could be used with other antigens of *B. pertussis* also. In addition, with modifications in culture conditions like in culture medium and time this assay could be used to measure antigen expression of other bacteria.

This assay was developed based on semi-quantitative ELISA, previously described by Barkoff et al., 2014 and was used to measure antigen expression status of clinical isolates of *B. pertussis*. In both, semi-quantitative and our new quantitative methods the specific monoclonal antibodies are accompanied with secondary antibodies in the indirect ELISA. However, with the semi-quantitative assay, the ACT of *B. pertussis* could not be detected. This may be due to the breakage of ACT after incubation period in high temperatures to lyse the bacteria or because of the low amount of ACT in the bacterial solution after a harvest from agar plate.

To solve the inability to measure ACT expression extra culture step on high-binding 96-well plates in Stainer-Scholte liquid medium in 35C, 150rpm was added, after the culture on Bordet-Gengou agar plate. With this culture step the ACT would not be neither lost in the agar plate but it would remain and bind to the wells nor break due to inactivation of bacteria. After this additional culture, the ACT was detectable with ELISA protocol. After optimization steps, 24h liquid culture pointed out to be adequate for this assay to reach measurable levels of virulence factors and prevent the saturation of the wells. The upper limit of detection of device used was OD-value of 4,0. The whole protocol will be described in below (See chapter 5.4).

5.2.1 Assay Specificity

Assay specificity was studied by comparing previously examined antigen expression background of *B. pertussis* isolates used in this study (data not shown) The expression background of the isolates was previously studied with assay introduced by (Barkoff et al., 2014; Heikkinen et al., 2008).

5.2.2 Assay Variability

To prove our ELISA based expression measurement consistent and precise inter- and intra-assay variations tests were performed. Inter-assay tests were performed by using five

independent wells/replicates containing same bacterial isolate and performed with each antigen. Intra-assay tests were performed using three different *B. pertussis* isolates in three independent expression tests for each virulence factor. In each individual expression test triplicate wells per virulence factor of isolate were used. CV was used to demonstrate the precision of the assay.

To assess the precision of introduced above, CV was employed to examine the precision of our ELISA based assay. We introduced acceptable CV% limit for inter and intra-assay measurements. For inter-assay measurements CV% of 15% and for intra-assay measurements CV% of 10% was considered as an upper limit of acceptance.

5.2.3 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, 3,33 µg, 5,0 µg, 6,66 µg, 8,0 µg and 10,0 µg) and the absorbance of each virulence factor was measured with plate reader (Victor Nivo™ multimode, Perkin Elmer, Turku, Finland) with 405nm wavelength. Concentrations of linear ranges were interpreted using pure antigens and with data not normalized.

5.3 Bacterial strain selection and cultures

Within the study period from 1991 to 2020, 904 clinical isolates of *B. pertussis* were isolated in Finland by Pertussis Reference Laboratory of the National Public Health Institute, 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. The isolates were grouped timely on 5-year time periods shown in Table 5. Study set comprised out of 156 isolates collected before introduction of aP (1991-2004) and 146 isolates collected after the introduction of aP (2005-2020).

The randomly selected isolates were cultured on Bordet-Gengou medium containing 15% sheep blood in 35°C for 72 hours. In addition, isolates were further cultured in 150µl Stainer-Scholte medium on microlon® high-binding microtiter plates (Greiner Bio-One GmbH, Frickenhausen, Germany) in shaker 35°C, 150rpm for another 24h. The isolates were inoculated to Stainer-Scholte medium to reach bacterial density of 0,05 +/- 0,01. After 24h culture bacterial density

reached from 0,1 to 0,20 accordingly. The Bacterial densities measured with plate reader (Victor Nivo^{TM} multimode, Perkin Elmer, Turku, Finland) 580nm.

5.4 Quantitative ELISA-based assay protocol for measuring virulence factor expression levels of *B. pertussis*

Detection of ACT, FHA, PRN, and PT expression levels was performed with specific indirect ELISA. The indirect ELISA protocol was performed on Microlon® high-binding standard ELISA microtiter plates (Greiner Bio-One GmbH, Frickenhausen, Germany) after 72h + 24h bacterial culture as described above. After the 24h liquid culture in duplicates the bacterial growth was measured and culture solutions were discarded and the wells were washed 3 times with 0,9% NaCl, 0,05% tween20 (Sigma, St.Louis, USA) washing buffer. Then antigen specific monoclonal antibodies in 1:1000 ratio in 2% skimmed milk were added 100µ/well and incubated 2h. After which, wells were washed, and the secondary antibodies were added in same dilution as monoclonal antibodies 100µ/well. After 2h incubation the wells were ones more washed, and the substrate (S0942-50TAB, Sigma, Helsinki, Finland) diluted in diethanolamine-MgCl-buffer (Reagena, Toivala, Finland) was added 100µ/well and incubated for 10 minutes in the dark and room temperature. Then the reaction was stopped with 3 molar NaOH solution (3 M NaOH, Merck, Espoo, Finland) 100µ/well and the absorbance of the wells was measured with plate reader (Victor Nivo^{TM} multimode, Perkin Elmer, Turku, Finland) with 405nm wavelength. The signal of deficient isolates remained under 0,25. To eliminate the effect of differentiate growth of bacteria and reach the most accurate results between measurements, the data gained from plate reader was normalized using following formula:

$$\frac{Virulence\ factor\ expression\ (OD)}{Bacterial\ growth\ (OD)} / Measured\ expression\ value\ for\ Tohama\ I.$$

Tohama I strain CIP81.32 and pure antigens provided by Peter Sebo, Institute of Microbiology of the Czech Academy of Sciences, Prague, CZ, were used as controls.

5.5 Determination of AC activity

For determination of AC activity and possible correlation between activity and expression of ACT, 16 isolates, half of the isolates isolated before 2008 and other half isolated after 2008 were randomly selected among tested 302 Finnish isolates and tested for AC activity parallel with ACT expression ELISA. AC enzymatic activity was measured by Peter Sebo's lab through formation of cAMP in presence of calmodulin described previously by (Ladant et al., 1986).

5.6 Comparison of virulence factor expression between isolates carrying different Fimbrial serotypes and ptxP genotypes

Groupwise comparison of virulence factor expression between Fim2/Fim3 and ptxP1/ptxP3 carrying isolates were performed to distinguish the possible association between FIM-serotype or ptxP-allele to the alterations in expressions in other virulence factors studied. The serotypes of isolates were examined previously by (Heikkinen et al., 2008; Niinikoski et al., 2024). Genotype profiles of isolates were routinely tested according to the protocol by Elomaa et al., 2005. For comparison between serotypes, virulence factor expressions between 162 FIM2 and 106 FIM3 were compared. For ptxP alleles, virulence factor expressions between 85 ptxP1 and 155 ptxP3 were compared. For other isolates tested within this thesis lacked the FIM-serotype or ptxP-allele information and were excluded from this comparison.

5.7 Statistical analysis

For measurement of virulence factor expression, 302 clinical pertussis isolates randomly selected out of 904 clinical isolates isolated by Finnish Reference Laboratory for Pertussis and Diphtheria in Turku, Finland within the study period were tested. The isolates were grouped timely on 5-year time periods as mentioned above and shown in Table 5.

For statistical analysis of virulence factor expression, achieved data for all virulence factors were assumed as non-parametric and statistical analyses for non-parametric variables were used. To examination of possible alterations of virulence factor expression within the study period Kruskal-Wallis test was performed. To distinguish difference between certain groups Mann-Whitney U -test also known as Wilcoxon rank sum test was performed.

The groupwise comparison of virulence factor 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.

To examine the significance of the correlation between expression of ACT and FHA the Spearman correlation was used.

In this thesis the GraphPad Prism 9.2.0 with a significance level of <0,05 was used.

6 Epilogue

This thesis sheds light on the alterations observed in *B. pertussis*, particularly after the introduction of aP vaccines. To be noted, early alterations took place in wP vaccine era. However, it has been observed that these alterations primarily occurred after the implementation of aP vaccines, with the main causative factor being the use of aP vaccines. The ability of *B. pertussis* to adapt quickly to changing circumstances is evident from the rapid occurrence of these alterations. Whether this is the main reason for resurgence of pertussis remains still to be discovered. However, this is one possible factor, with increasing evidence, behind the resurgence and simultaneously the negative side of current aP vaccines including only limited components.

Nevertheless, aP vaccines are effective and remarkably safe approach to prevent pertussis. However, to prevent and restrain the further resurgence, actions need to be taken. Encouragingly, approaches to improve the prevention of pertussis are ongoing. Altogether, it is crucial to conduct research on the rapidly adaptative *B. pertussis* besides the vaccine development to achieve and retain up to date information of circulating strains and effective prevention of the pertussis in the future.

Further research is needed to uncover the full consequences of these alterations and their implications for vaccine development. Understanding the associations between the reported alterations and the interplay between virulence factors will contribute to our knowledge of *B. pertussis* pathogenesis and aid in the development of more effective vaccines against pertussis.

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8 List of abbreviations

ACD	adenylate cyclase domain
ACT	adenylate cyclase toxin
aP	acellular pertussis
AR	acylation region
AU	arbitrary unit
BrkA	*B. pertussis* resistance to killing antigen
Bvg	*B. pertussis* virulence gene
CV	coefficient of variation
DtaP	diphtheria-tetanus and acellular pertussis vaccine
ECDC	European Centre for Disease Prevention and Control
ELISA	enzyme linked immune sorbent assay
FHA	filamentous hemagglutinin
FIM	fimbria (serotypes 2, 2.3 and 3)
Hly	hemolysin
HR	hydrophobic region
NIP	national immunization protocol
NPA	nasopharyngeal aspirates
NPS	nasopharyngeal swaps
OD	optical density
OMV	outer membrane vesicles
PRN	pertactin
PT	pertussis toxin
ptxA	pertussis toxin S1 subunit
ptxP	pertussis toxin promoter
R0	the basic reproduction number
RGD	arginylglycylaspartic
RTX	repeats in toxin
Tdap	diphtheria-tetanus and acellular pertussis booster vaccine
TPS	two-partner secretion
TR	translocation region
WHO	World Health Organization
wP	whole cell pertussis vaccine

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