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Bordetella pertussis: clinical and molecular trend in the decade 2010-2019

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SUMMARY

Pertussis or whooping cough is a bacterial disease that consists in severe infection of the respiratory tract mainly caused by *Bordetella pertussis*. The disease is especially dangerous for young infants and spreads easily from person to person, mainly through droplets produced by coughing or sneezing [1,2]. For a long time pertussis has been one of the most common childhood diseases worldwide, but the introduction of infant whole-cell vaccination (WPV) in the 1950s led to a steep decline in the number of pertussis cases and deaths in children. [1] Due to the presumption that the whole cell vaccine was not completely safe, from the 1980s many countries started to replace it with the acellular vaccine (ACV) [3]. Despite the fact that the vaccination schedule has little differences between countries, either in the timing of administration of the vaccine or in the type of vaccine used, all the developed countries have high childhood primary immunization rates [4]. Because of that, the resurgence of disease observed from the beginning of 1990s was unexpected. As shown in Palazzo et al. studies, the pertussis cases are increasing also in Italy [14] and particularly, in Tuscany [16]. In Tuscany, the diagnosis of pertussis infection is carried out at the Immunology Laboratory of the Meyer Children's Hospital. We analyzed all cases of pertussis diagnosed from 2010 to 2019 included in the National Register of Invasive Bacterial Diseases in order to categorize them according to the age of onset of symptoms, vaccination status and gestational age. On the basis of these data, it emerged that the subjects most affected are the youngest children, especially those under the year of life and in particular infants in the first three months of life. Therefore, any effort aimed at increasing awareness of the practice of vaccination in pregnancy is essential, as it could be the only effective method to reduce pertussis among infants under 3 months of age [98]. These subjects are not only those who have the greatest risk of getting sick, but also have the greatest risk of presenting with severe infections that require hospitalization. Therefore, we have analyzed the trend of hospital admissions taking into account the vaccination status and age of patients. Reducing hospitalization rates means significantly reducing public health costs, so we performed a pharmaco-economic analysis to compare the impact of hospitalization costs with the costs of a correctly followed vaccination strategy. In addition, our studies have shown that most children with whooping cough do not have adequate vaccination coverage. We have also shown that in Tuscany the decline

in vaccination coverage corresponds to a significant increase in cases of whooping cough. However, the increase in pertussis can be caused not only by reduced vaccination coverage, but also by pathogen adaptation. In countries where hexavalent vaccination is widely used, there are variations between the antigens of the strains used in vaccine production and those of the circulating strains [27,28]. For this reason we have studied allelic variations in genes associated with virulence: genes for the promoter of pertussis toxin (ptxP), for subunit A of pertussis toxin (ptxA), for pertactin (prn). Our analysis conducted on the strains of *B.pertussis* in circulation from 2010 to 2019 has shown that the allele structure of circulating bacteria no longer corresponds, at least for some proteins, to the composition of available vaccines. The consequence is therefore that the vaccination protection may be reduced.

CONTENTS

INTRODUCTION

WHOOPING COUGH

Whooping cough is a gram-negative bacterial, highly contagious, acute respiratory disease caused by *Bordetella pertussis* that is a strict human pathogen with no known animal or environmental reservoir. While nine species of Bordetella have been identified to date, only three additional members, *B. bronchiseptica*, *B. parapertussis*, and *B. holmesii*, have been associated with respiratory infections in humans and other mammals [5,6]. They express a number of virulence factors including pertussis toxin (Ptx), filamentous haemagglutinin (Fha), pertactin (Prn), (Fim) type 2 and type 3 fimbriae, adenylate cyclase toxin (act), tracheal cytotoxin (Tct) and lipooligosaccharide (LPS). The pathogenesis of pertussis is incompletely understood, but Fha, Prn and Fim facilitate attachment to the epithelial cells, and Ptx, Tct and act allow evasion of host immune factors and destruction of the epithelial cells [1]. In 2013, according to WHO estimates, pertussis was still causing around 63000 deaths in children under 5 years of age worldwide, although there is considerable uncertainty about these estimates in view of the scarcity of reliable surveillance data, especially in developing countries [1]. The young children are indeed those for whom the disease can be more dangerous. Apart from the patient age, several factors known to affect the clinical manifestations of *B. pertussis* include previous immunization or infection, presence of passively acquired antibodies, antibiotic treatment, number of bacteria at exposure, host genetic and acquired factors and the genotype of the bacterium [6]. The classic symptom of pertussis, from which the original name “whooping cough” is derived, consists of violent and rapid coughing with rapid expulsion of air from the lungs, following which the patient is forced to inhale with a loud “whooping” sound. *B. pertussis* infection includes four phases: starting from the incubation period that usually lasts seven to ten days, even if incubation periods as long as four weeks have been observed as well. The clinical course of pertussis progresses through the catarrhal stage, lasting one week, in which infants and children often present with normal body temperature or low-grade fever, malaise, sore throat, nasal congestion, rhinorrhea, lacrimation, sneezing, and mild progressive dry cough. The third stage of illness, the paroxysmal stage, is a three to six weeks long phase, with intense and violent coughing that lasts several minutes and is

variably associated with cyanosis, eye proptosis, tongue protrusion, salivation, thick oral mucus production, lacrimation, engorgement of neck veins, vomiting, fatigue, and respiratory exhaustion. The classic sign of pertussis, the inspiratory whoops, manifests at this stage. Such paroxysms often occur at night and increase in frequency during the first one to two weeks of this stage through weeks two to three, with a gradual decline thereafter. In the last stage, convalescence, coughing paroxysms recede in frequency, duration, and severity. However, a mild, chronic, nonparoxysmal cough can last up to 6 weeks. In its early catarrhal stage, pertussis is highly contagious and untreated patients may transmit infection for three weeks or more following the onset of typical coughing attacks, although communicability diminishes rapidly after the catarrhal stage. In infants, pertussis may present three types of severe complications: pulmonary, neurologic and nutritional complications. Therefore, pertussis may cause hospitalization and even death in infants too young to be vaccinated. Most adolescents and adults have symptoms that are milder than those of infants and children, instead. Consequently, pertussis in adolescents and adults can even escape detection by clinicians [7]. Moreover, numerous studies have shown that adults and adolescents provide a reservoir of *B. pertussis* and are the major source of transmission to partially immunized infants and children [6].

EPIDEMIOLOGY

Pertussis is an endemic disease in developing and developed countries, with frequent outbreaks occurring every two to five years at different places around the world. It occurs mostly during the summer and autumn, although still present throughout the year.

It is important to say that to estimate the global pertussis disease burden is very difficult: as a matter of fact, the World Health Organization has noted that the application of a standardized set of pertussis case definitions within an overall surveillance framework has not been uniform.

Before vaccines became widely available in the 1950s, pertussis was one of the most common childhood diseases worldwide [1,7].

The incidence of pertussis has been greatly reduced by mass vaccination, despite that the three to five yearly peaks of disease outbreak observed in the pre-vaccine era are still noticed.

In these last decades, it was realised that mild or atypical pertussis is common, and notably, also in adults showing with chronic cough, this shift in the age distribution of pertussis towards older age groups, adolescents and young adults, has been reported in some high-income countries, in particular where ACV vaccines have replaced WPV vaccines for primary vaccination series. The age shifts may be explained in part by an increasing recognition of the less typical disease manifestations in older subjects, more sensitive laboratory testing, and more sensitive surveillance extended to cover the entire life span. Waning of vaccine-derived protection caused by adaptations of *B. pertussis* is also thought to increase the susceptibility of adolescents and adults [1].

Indeed, significant changes in *B. pertussis* populations have been observed in the latest years, suggesting a potential role for pathogen adaptation in the persistence and resurgence of pertussis. These changes include antigenic divergence with vaccine strains and increased production of pertussis toxin. Antigenic divergence will affect both memory recall and the efficiency of antibodies, while higher levels of pertussis toxin may increase suppression of the innate and acquired immune system [4].

GLOBAL EPIDEMIOLOGY

Before vaccines became widely available in the 1950s, pertussis was one of the most common childhood diseases worldwide. Limited early data indicated that most individuals were infected in childhood, of whom >50% developed clinical disease. According to prevaccine data from the United States of America (USA), approximately 80% of cases occurred in children <5 years of age and less than 3% of cases in subjects aged ≥15 years. Similar patterns were reported in prevaccine studies from developing countries, including in Africa and southern Asia. Case fatality rates (CFR) were and remain highest in infancy [8]. Historic early data were likely biased towards the more severe presentation of pertussis disease and may not reflect the full burden of milder disease across all age groups.

The introduction of effective infant vaccination programmes was associated with a steep decline in the number of pertussis cases and deaths in children. Pertussis vaccine (combined with diphtheria and tetanus toxoids) has been used in the Expanded Programme on Immunization (EPI) since its inception in 1974. It was estimated that without vaccination there would have been >1.3 million pertussis related deaths globally in 2001 [9]. In 2013, according to WHO estimates, pertussis was still causing around 63,000 deaths in children aged <5years [10] although there is considerable uncertainty over these estimates in view of the paucity of reliable surveillance data, particularly from developing countries. In 2014 global vaccination coverage with 3 doses of a pertussis-containing vaccine was estimated at 84% [11].

In 2018, WHO estimates 151,074 pertussis cases globaly and 86% Diphtheria-tetanus-pertussis immunization coverage (DTP3) [11] (figure 1).

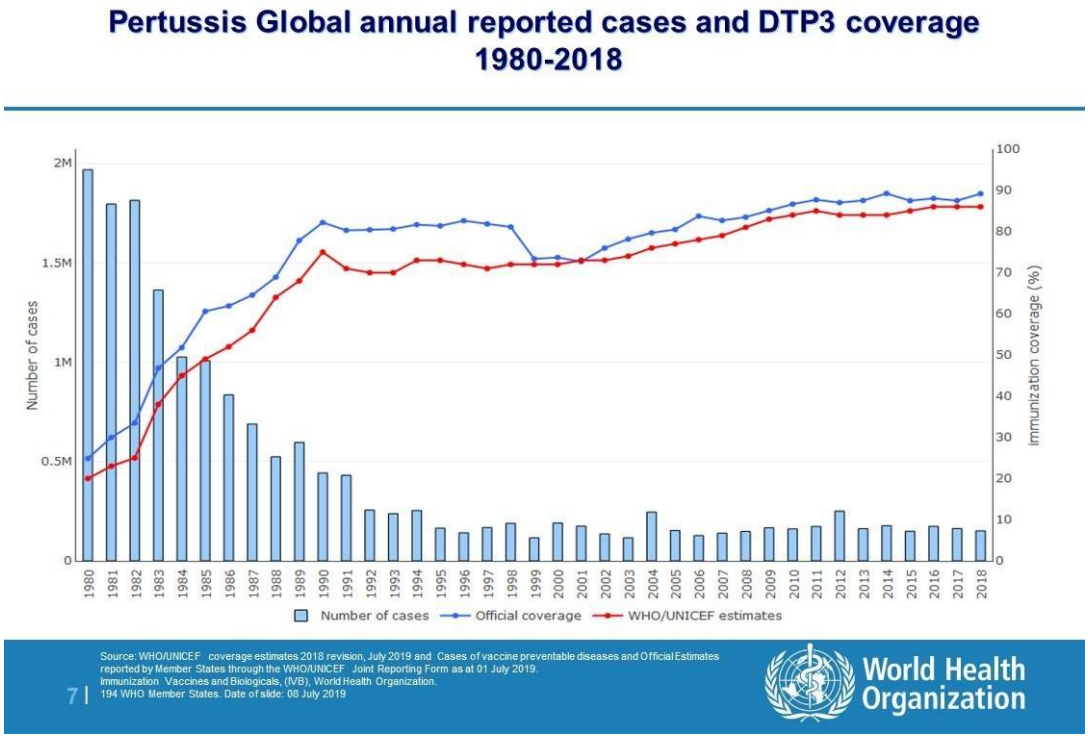


Figure 1. Pertussis Global annual reported cases and DTP3 coverage 1980-2018.

EUROPEAN EPIDEMIOLOGY

In 2017, 42.242 cases of pertussis were reported in EU/EEA countries. Five countries (Germany, the Netherlands, Poland, Spain and the UK) accounted for 76% of all notified cases (figure 2). The notification rate in 2017 was 9.4 cases per 100 000 population, which was in the same range as for the last three years. The overall notification rate remains above the pre-epidemic levels seen before 2012, the year in which a substantial increase was observed in many Member States. Individuals ≥ 15 years of age accounted for 62% of all cases reported. Infants below the age of one year, too young to be vaccinated, were the most affected age group, with the highest rate of 53.9 per 100,000 population and 3 deaths reported, followed by the rates in 10–14-year-olds (25.0 cases per 100 000 population). The clinical presentation of pertussis in adolescents and adults may be mild and is often not recognised. This poses a transmission risk to infants who are too young to have completed the primary pertussis vaccination series. The objectives of pertussis prevention and control include prevention of severe disease and deaths among infants < 6 months of age through well-adapted and implemented vaccination programmes [12].

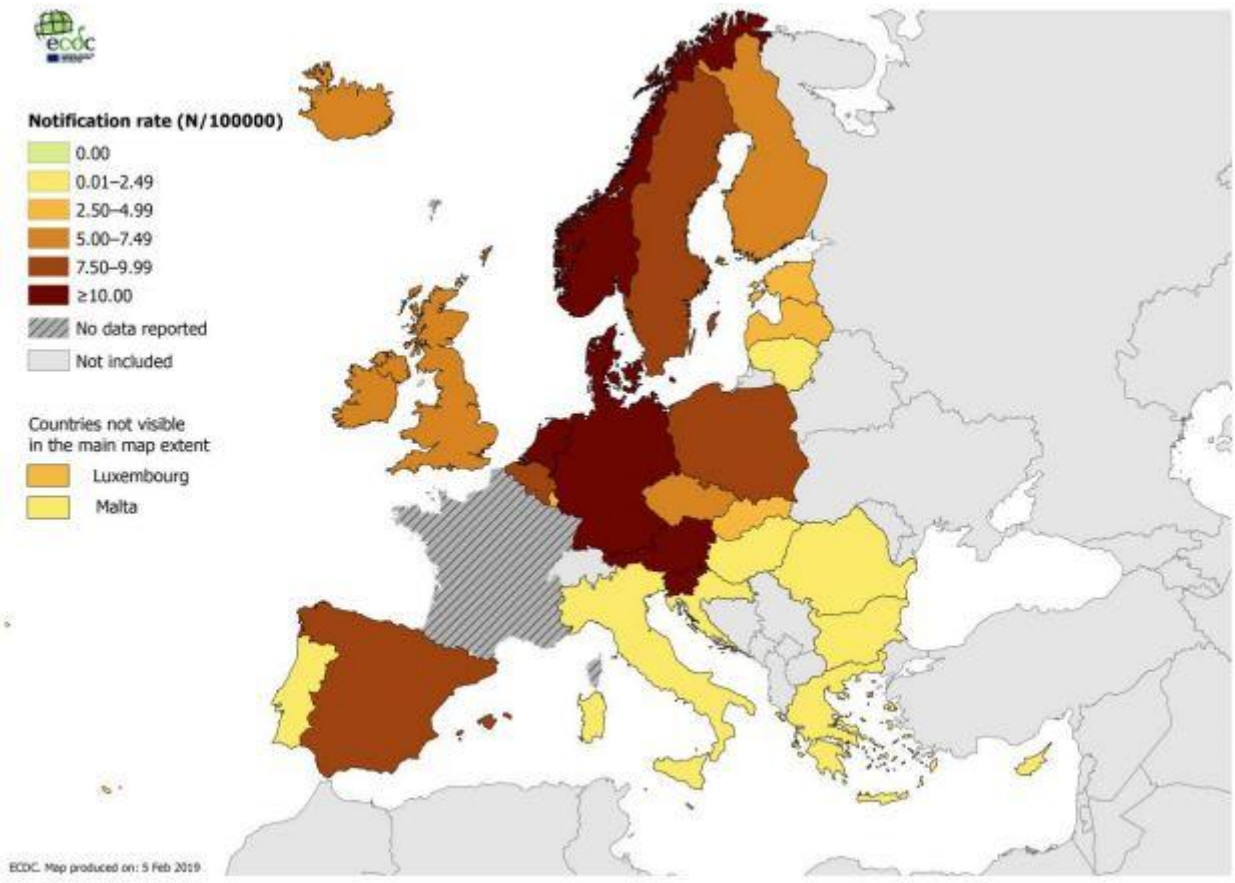


Figure 2. Distribution of pertussis cases per 100 000 population by country, EU/EEA, 2017.

ITALY'S EPIDEMIOLOGY

In Italy, since 1962, the Ministry of Health has recommended vaccination against pertussis with cellular vaccine; however, until 1991 the coverage rate did not exceed 40% and consequently was insufficient to control the spread of the infection. The epidemiological trend has changed accordingly to the coverage rates of pertussis vaccine achieved in different periods. With the introduction of the whole cell vaccine, pertussis incidence showed a decreasing trend from 76.2 per 100,000 population in 1961 to 12.7 in 1981, although immunisation coverage was very low, with reported figures ranging between 10 and 16% during the years 1974 to 1981 (figure 3). Epidemic cycles continued to occur every three to five years. An incidence peak was observed in the period from 1983 to 1987, reaching values of 45.6 per 100,000 population in 1983 and 48.2 in 1987. Then, starting from the 1994, when acellular vaccines was made available and included in combined products, vaccination coverage has significantly improved, leading to register a lower incidence rates and a relevant decrease of complications and mortality in the first year of life and in the pre- school age. The coverage rate with three doses in children aged 12–24 months was equal to 88% in 1998, increased to 95% in 2003, reaching 96.6% in 2008 and consequently in 1998, pertussis cases notified were almost 7,000; while in 2005, notifications were 802 and during the following years this number decreased further. Moreover, data from 2010-2013 reported a 97.6% drop in disease burden.

The vaccination schedule currently adopted in Italy consists of three doses of acellular vaccine administered at 3, 5 and 11–12 months of age [13].

To date, however, the number of pertussis cases is steadily increasing in children, adolescents and adults[12].

Children <1 year of age continue to be the age group with the highest incidence rates but the disease is increasingly affecting adolescents and adults [193].

In Italy, pertussis is a mandatory notifiable disease; but the case definition is based only on clinical diagnosis. Consequently, the detection of several cases of atypical adult pertussis may be misdiagnosed. Therefore, together with clinical symptoms of pertussis, the use of laboratory techniques is desirable in order to make the national incidence figures more valid. However, Palazzo et al. comparing the seroprevalence of IgG antibodies to pertussis toxin in selected adult age groups (in the age groups 20–29, 30–39 and >60 years and from

clinical laboratories in five Italian regions), with the use of sera collected in 2012–2013 and in 1996–1997, show an infection increase from 9.3 % in 1996–1997 to 14.1 % in 2012–2013. Their data clearly indicate a

significant increase in the circulation of *B. pertussis* in adults in Italy; therefore, they showed that the statutory notification system underestimates the real incidence of the disease and that adult age groups can serve as a reservoir of *B. pertussis* and be responsible for onward transmission to vulnerable infants [14, 193].

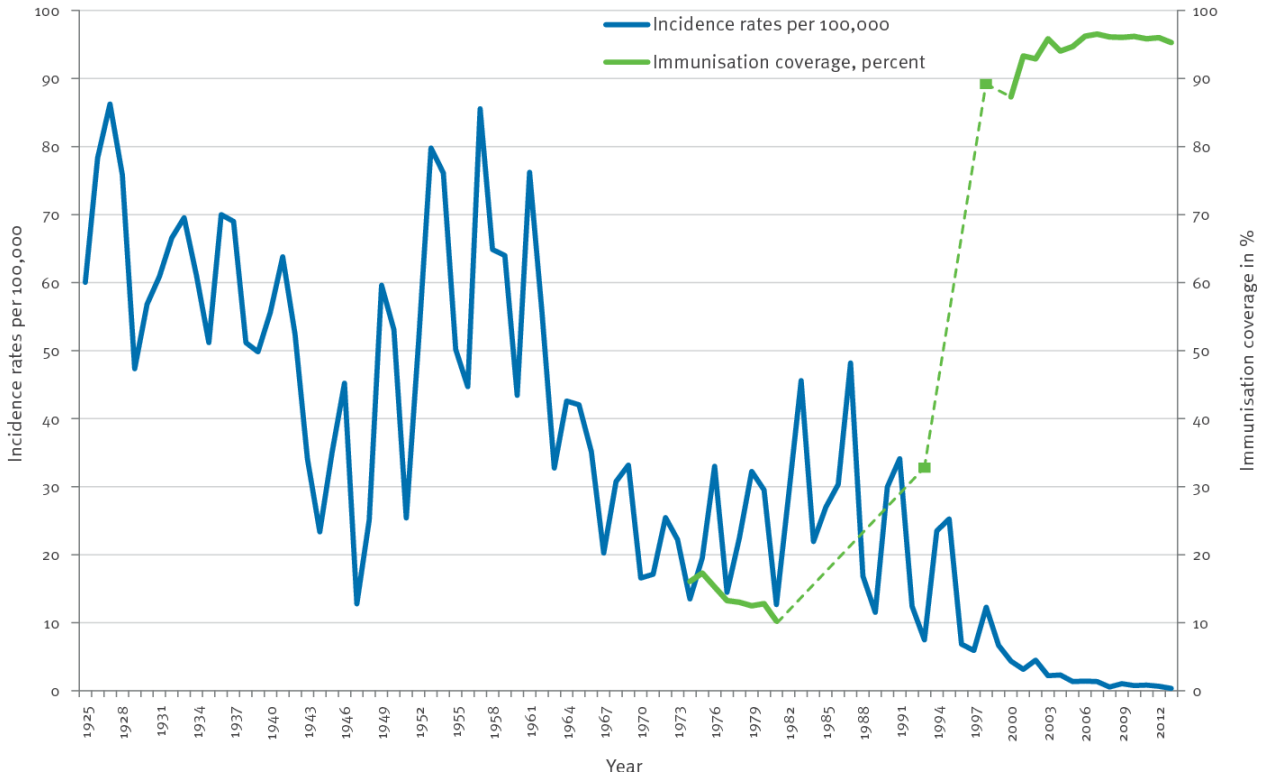


Figure 3. Pertussis incidence and pertussis immunisation coverage at 24 months, Italy, 1925-2013.

TUSCANY 'S EPIDEMIOLOGY

In 2014 in Tuscany, the overall hospitalization rate increased reaching 5.83 [95% confidence interval (CI): 3.84–7.82] and 5.04 (95% CI: 3.21–6.88) cases per 100,000 in 2013 and 2014, respectively. A dramatic significant increase in hospitalization rate was observed in infants up to

95.7 per 100,000 in 2014. A parallel decrease in vaccine coverage rate (VC%) in children was noticed in the same period (95.6%). The 95.6 VC% observed in 2014 was still higher than the 90%– 92% threshold recommended by the World Health Organization, but could be a contributing factor to pertussis resurgence [15,16]. To date the pertussis vaccine coverage rate has further decreased at 94.4% [192].

BORDETELLA PERTUSSIS

Bordetella pertussis was first isolated in pure culture by Bordet and Gengou in 1906. It is a small (approximately 0.5 x 1 µm) encapsulated, strict aerobe, non-motile and non-sporulating bacterium.

Following the inhalation of infected aerosol, *B. pertussis* enters the upper respiratory tract and adheres to ciliated epithelial cells in the nasopharynx and trachea. Once attached to the mucosal surface, *B. pertussis* produces a wide array of virulence factors, including adhesins and toxins.

The concerted expression of these factors prevents rapid clearance of the bacteria and enables replication and dissemination to the lower areas of the respiratory tract [17].

The expression of the majority of these virulence factors is under the control of a two component regulatory system encoded by the bvgAS locus [18].

BvgS is a 135-kDa transmembrane sensor kinase consisting of several domains involved in phosphorylation cascade, while BvgA is a 23-kDa response regulator protein, with a receiver domain at its N-terminus and a DNA-binding domain at its C terminus [19].

Under inducing signals BvgS autophosphorilates and initiate a phopshorelay that leads to BvgA phosphorylation and activation [20].

Activated BvgA dimerizes and binds to specific DNA sequences to positively or negatively regulate transcription [21].

In response to environmental conditions, BvgAS controls the expression of at least three distinct phenotypic phases, each characterized by maximal expression of some genes and minimal expression of others [22].

The signals to which BvgS responds in vivo are still unknown.

Bvg+ phase occurs when bacteria are grown under permissive conditions. In this phase BvgAS is active and promotes the expression of virulence activated genes (vag-genes, divided in class 1 and class 2 genes), while represses the expression of virulence-repressed genes (vrg-genes, or class 4 genes) by the repressor protein BvgR, whose gene is located downstream of the bvgAS locus and is activated by BvgA [23].

Vag-genes encode adhesins and toxins that play a key role in the respiratory tract colonization in vivo [24].

Bvg- phase occurs when bacteria are grown under modulating conditions, such as growing at low temperature (25°C) or in the presence of nicotinic acid or MgSO4 [25].

During this phase, BvgAS is inactive and is unable to repress vrg-genes, which are therefore maximally expressed, while no expression of vag-genes occurs. Since these genes are expressed when bacteria are grown under limiting conditions, they may be involved in *B. pertussis* survival in the ex vivo environment. Bvgi phase occurs when bacteria are grown under submodulating conditions (low concentration of chemical modulators). In this phase bacteria display a phenotype that is intermediate between Bvg+ phase and Bvg- phase, since BvgAS is partially active: vrg-genes and class 1 genes (e.g. PTX, ACT) are not expressed, while class 2 genes (e.g. FHA) and the genes specific of this phase (class 3 genes) are maximally espresse [26].

This phenotype may have an important role in transmission between hosts, but this remains to be further elucidated. The complexity of this system may allow multiple levels of control of gene expression that could be important in the infection cycle.

B. pertussis virulence factors include several adhesins and toxins that contribute to its ability to cause the disease. The major virulence factors and antigenic components expessed during the virulent Bvg+ phase, are schematically reported (figure 4). Among them are the antigens included in acellular pertussis vaccine.

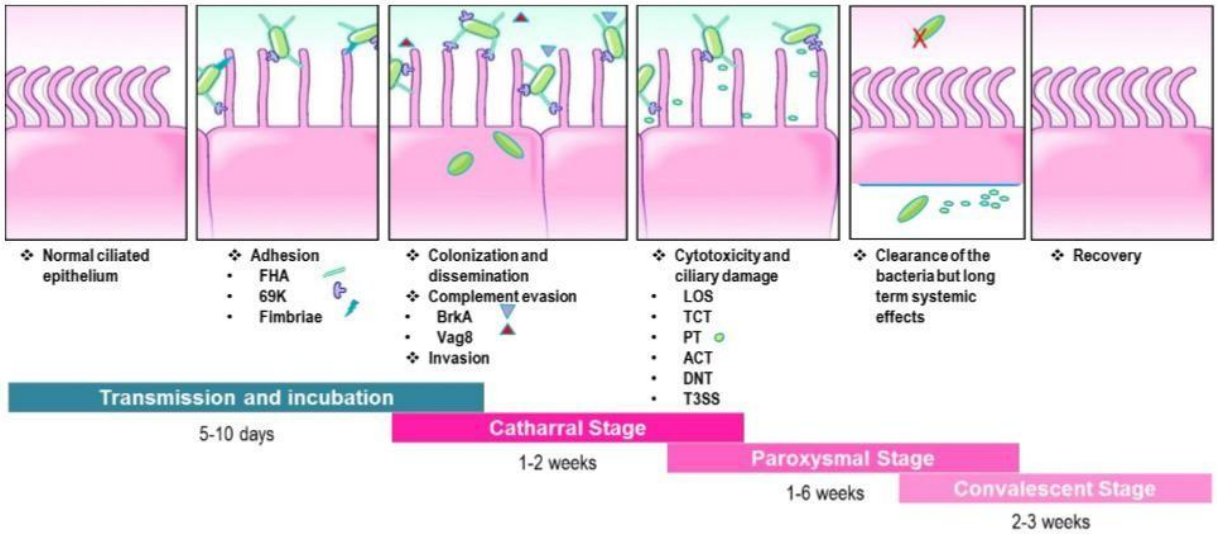


Figure 4. *B. pertussis* pathogenesis: transmission and disease progression Adapted from [29]. *Bordetella pertussis* engages multiple strategies in order to create a niche for colonization of the mucosal epithelium. From left to the right: several virulence factors mediate adhesion to respiratory cells (FHA, 69K, Fimbriae), colonization and dissemination. *Bordetella pertussis* evades the immune system and the complement mediated killing (BrkA and Vag8) and invades epithelial cells. Tracheal cytotoxin (TCT) and lipo-oligosaccharide (LOS) synergistically evoke ciliary damage to the respiratory epithelium. Pertussis Toxin (PTX), Adenylate cyclase toxin (ACT) and the type III secretion system (T3SS) subvert intraepithelial signaling pathways leading to cytotoxicity.

BORDETELLA PERTUSSIS VIRULENCE FACTORS

Bordetella pertussis (figure 5), was first cultured by the scientists Jules Bordet and Octave Gengou in the beginning of the 20th century. In 1909, a thorough study of the bacterium was published by Martha Wollstein – the first female member of the American Pediatric Society. She summarised the findings of Bordet and Gengou as follows: "The Microbe de la Coqueluche is short, poled, ovoid, a trifle larger than Bacillus influenzae [today called Haemophilus influenzae], not motile, very regular in size and shape, arranged singly within groups, occasionally in pairs, either end to end or parallel. It is negative to Gram's stain, but colors especially well with toluidine-methylene blue". In addition, she already reported that "the bacillus of Bordet and Gengou is present in the sputum in early cases of pertussis, and in the lungs at autopsy in fatal cases of the disease. After the second week it is not present in the sputum in sufficiently large numbers to be readily isolated." [30] Human are the sole host and reservoir of *B. pertussis*. Since Bordetellae are inhibited by several agents such as fatty acids, metal ions and peroxides, a special culture medium containing protective agents such as charcoal, blood or starch is needed to isolate them. Cephalexin is often added to the culture medium to suppress the growth of other bacteria. The most commonly used media are Bordet-Gengou [31] and Regan-Lowe [32], where *B. pertussis* grows as round, mercury-silver colored and shiny colonies. In addition, *B. pertussis* shows haemolytic activity on Bordet-Gengou agar. Both in vivo and in vitro, *B. pertussis* produces virulence factors such as adhesins and toxins (figure 5) [33,34].

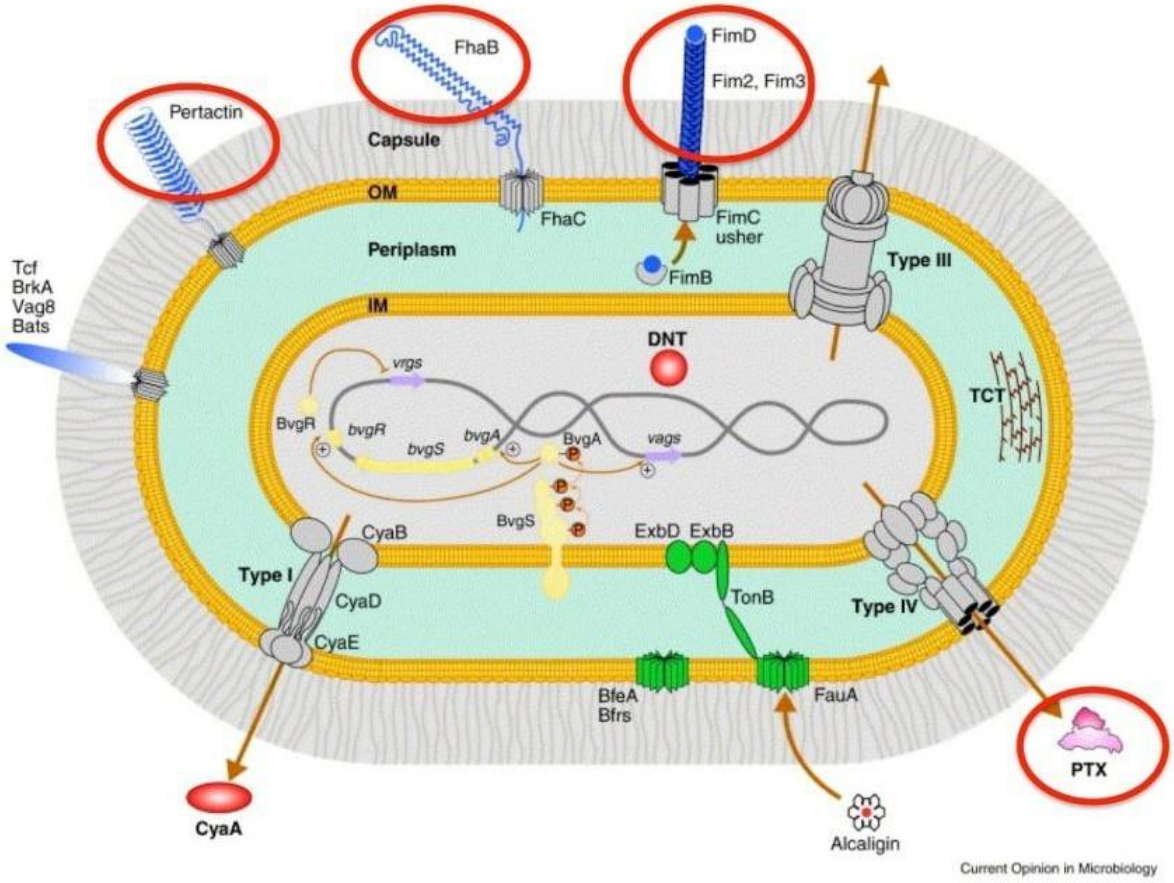


Figure 5.[35] The major virulence factors and antigenic components expessed by *Bordetella pertussis*. Circled in red: are the antigens used in the acellular vaccine (ACV).

TOXINS

Well-known toxins produced by *B. pertussis* are pertussis toxin (PTX), adenylate cyclase toxin (CyaA), dermonecrotic toxin (DNT) and tracheal cytotoxin (TCT). CyaA, DNT and TCT are expressed in *B. pertussis*, *B. parapertussis* and *B. bronchiseptica*. PTX is only expressed in *B. pertussis* even though its genes are present in all three species. There is wide variability in the roles of the toxins in the infection and virulence of *B. pertussis*. PTX is the only one of the four toxins known to contribute to the attachment of the bacterium to the epithelium of the host. PTX, CyaA and DNT cause systemic effects; PTX, CyaA and TCT have a role in the evasion of the host defence. In addition, all four toxins induce local effects on the respiratory epithelium of the host. The two most widely studied toxins PTX and CyaA are discussed below (figure 5) [36, 37]. PTX, CyaA, DNT and TCT are not the only toxin, *B. pertussis* produces also lipopolysaccharide (LPS) which is an endotoxin and is described below.

PTX is a protein composed of hexameric AB_5 structure and it is 105 kDa in size. AB toxins, like PTX, cholera toxin and diphtheria toxin are ADP-ribosylating toxins [43]. PTX hexamer consists of a catalytic subunit referred as A or S1 subunit and B_5 subunit consisting of S2, S3, two S4 and S5. Subunits S1 – S5, in order of decreasing size, are produced by the genes ptxA to ptxE [44,105]. S2 – S5 form a pentamer, including two S4 subunits. The B heteropentamer has no enzymatic activity, but is required for efficient binding of the toxin to cells.

The binding site consists of two dimers, S2S4 and S3S4, joined by S5 [38]. The genetic alteration of S2 and S3 suggest that the two subunits can substitute each other to a certain degree, but their main biological activities are different. If S3 is replaced by S2, the toxin production is significantly enhanced; if S2 is replaced by S3, the ADPribosylation activity of the toxin is increased [39]. PTX is a soluble toxin secreted to the extracellular space by type IV secretion system (T4SS) named as pertussis toxin liberation (Ptl) [40]. Both the ptx and ptl genes are located in the same operon and expressed from the same ptxP promoter enabling the efficient production and secretion of PTX [41].

PTX is able to bind to almost all cell types and ligands are typically glycoproteins with sialic acid but glycosylation is not a prerequisite for binding [45,46]. PTX enters the cytosol of the

host cells and in the cytoplasmic membrane the S1 subunit ADPribosylates the guanine nucleotide protein (G protein), inactivating it [45]. This G protein inactivation causes the accumulation of cyclic adenosine monophosphate (cAMP), an important messenger in the cell signaling, leading to dysregulation of immune responses. This causes a variety of biological actions, depending on the targeted cells, such as histamine sensitization, leukocytosis, lymphocytosis and delayed recruitment of neutrophils to the respiratory tract [42,47,48]. PTX is currently considered as a facilitating factor for adhesion, but its main function is the modulation of host immune responses [106,46,107]. Antibodies against PTX are important for the protection against pertussis and that is why PTX is included, alone or in combination with other antigens, in all currently used acellular vaccines [45,49]. Nevertheless, similar to the strains not expressing FHA and PRN, also *B. pertussis* strains not expressing PTX have been reported [108].

CyaA is a single 200-kDa polypeptide secreted by type I secretion system (T1SS) [50] and composed of two protein moieties: an adenylate cyclase enzyme and cytolysin [51]. It has a dual role in manipulating the physiology of the host cell as it intrudes into both the cellular signalling pathways and the ion homeostasis of the host cell. After the CyaA is secreted, it binds to the target cell membrane in two conformational isoforms with different functions: the membrane translocator promotes the release of the adenylate cyclase domain to the target cell cytosol, and the cation-selective pore composed of two CyaA molecules represents the cytolysin activity of CyaA [52]. CyaA is known to target neutrophils, phagocytic cells that migrate to the site of infection [53]. It is suggested that CyaA acts in proximity to the bacteria producing it – contrary to the freely soluble PTX – and that it intoxicates the neutrophils on the site of infection: in the early stage of B. pertussis infection the influx of neutrophils is delayed by PTX, and after that, CyaA promotes the infection by depleting the neutrophils [54]. The role of CyaA and its mechanisms of action have been well reviewed by Jana Vojtova and colleagues [55]. CyaA is not included in any of the currently used acellular vaccines.

Lipopolysaccharide LPS is a characteristic and dominant element of the outer membrane of gram- negative bacteria, likewise *B. pertussis* and it is also an endotoxin. The basic structure

of LPS consists of a lipid A molecule and a two-part polysaccharide; the core oligosaccharide and a polysaccharide protruding outward the bacterial membrane are called O-antigen [112]. In *B. parapertussis* and *B. bronchiseptica*, LPS includes the O-antigen whereas in *B. pertussis* LPS is lacking the O-antigen structure [113]. The LPS absent of O-antigen is commonly called LOS. Because of the LPS' wide prevalence in gram-negative bacteria, host immune defense senses it easily by molecules such as surfactants and toll-like receptor 4 (TLR4) [114,115].

However, B. pertussis is able to evade this recognition via surfactant due to the lacking of O- antigen. Whereas for TLR4 recognition, the lipid A moiety of LPS is the main domain identified [116].

ADHESINS

Bacterial components mediating the adherence of the bacteria to the host are called adhesins. They are often located on the surface of the bacteria, and are important for the attachment and colonisation. The main adhesins of *B. pertussis* are filamentous hemagglutinin (FHA) [56], fimbria (Fim) [57] and an autotransporter protein pertactin (Prn) [58].

Other autotransporters – proteins that mediate their secretion through the outer membrane themselves – such as tracheal colonisation factor (TCF) [59, 60], BrkA [61] (encoded by brkA gene in the locus called the Bordetella resistance to killing, brk) and Vag8 [62] (encoded by viractivated gene-8, vag-8) have also shown adhesive properties. The three most widely studied adhesins FHA, Fims and Prn are discussed below (Figure 4).

FHA is suggested to act as an immunomodulatory and immunosuppressive agent, and it is considered to be the most important factor in the attachment of *B. pertussis* [63]. It is synthesised as a 367 kDa precursor encoded by the fhaB gene, and secreted as a monomeric 220 kDa protein to the outer membrane and extracellular space [64, 65, 66, 67]. FHA is secreted by a type V secretion systems/two-partner secretion (T5SS/TPS) pathway [68, 69]. Several different binding motifs of FHA refer to its important role in the adhesion, and the secretion of free FHA appears to contribute to bacterial colonisation [70]. In addition, FHA has been shown to be a crucial factor in the biofilm formation of *B. bronchiseptica* [71, 72] and of *B. pertussis* [73].

In addition to adhesion, FHA functions as an immunomodulator that interferes with the host immune responses against *B. pertussis*. As a matter of fact, it has been described in that FHA suppresses the pro-inflammatory interleukin (IL)-12 production by increasing the expression of IL- 10 [75, 109]. FHA also mediates proapoptotic response in monocytic and bronchial epithelial cells in vitro [110]. Despite the critical role of FHA as an adhesive factor and immunomodulator, very recently, *B. pertussis* isolate not expressing FHA has been observed in France[111].

Besides PTX, FHA is the primary component of the acellular pertussis vaccines [74, 49].

Fims are filamentous cell surface structures used by the bacteria to adhere to one another and to the host cells. They are composed of a minor 40-kDa subunit, FimD, and a major

subunit, 22-kDa Fim2 or 22,5-kDa Fim3, corresponding to the serotypes Fim2 and Fim3, respectively; serotype Fim2.3 produces both Fim2 and Fim3 fimbriae [76, 77].

The subunits FimD, Fim2 and Fim3 are encoded by fimD, fim2 and fim3 genes, respectively, and play their own roles as adhesins [78, 79, 80]. The serotype expressed by the bacteria depends on the phase-variable expression of fim2 and fim3 on the transcription level; this regulatory mechanism is called phase variation [81]. In addition to fimD, fim2 and fim3, a silent fimbrial subunit gene, fimX, has been described in the *B. pertussis* genome [82]. Besides the adhesive activities, Fims interfere in the immune response of the host as an anti-inflammatory agent [83]. Despite Fim being an important virulence factor of *B. pertussis*, it is not included in most of the acellular vaccines [84]. However, more recently, anti-fimbriae antibodies have been shown to be important for the protection provided by both whole-cell [100] and acellular vaccines [101]. Thus, these antigens should be carefully considered as components of any new pertussis vaccines.

Prn, also called P.69 protein, is a 69-kDa autotransporter protein located on the outer membrane of *B. pertussis* [85, 86, 87]. It is synthesised as a 93,5-kDa precursor, and secreted to the outer membrane by an autotransporter pathway of T5SS [88, 87, 89]. Prn has been shown to act as an adhesin [90]. Even though the mechanisms of action and the role of Prn during the infection are not yet fully understood, its role as a vaccine component is well justified: in fact the opsonic anti-Prn antibodies were found to be essential for the antibody-mediated phagocytosis of *B. pertussis* [91].

The rise of *B. pertussis* not expressing Prn has been observed in many countries such as Japan, USA, Australia, France, Finland, the Netherlands, Sweden, Norway and Israel [117, 118, 119, 120, 121, 122]. These evidences support the dispensable role of Prn for *B. pertussis* and the adaptation of *B. pertussis* to vaccinated populations in countries that use aP vaccine.

SECRETION SYSTEMS FROM TYPE I TO VI

The Gram-negative bacterial pathogens secrete proteins across the inner membrane, periplasm and outer membrane to the extracellular space or into the host cells via several secretion systems; six of those have been described and named as type I to VI secretion systems (T1SS to T6SS) [123]. Within these secretion systems, proteins are translocated across the inner membrane in the unfolded form via the general secretion route, Sec-pathway, or in the folded form via the twin- arginine translocation, Tatpathway [124]. Via direct secretion by T1SS, CyaA is secreted from the bacterial cytoplasm to the extracellular space. The T1SS for CyaA secretion is composed of three proteins, CyaB, CyaD and CyaE (figure 5) [125]. T2SS is a Sec-dependent pathway, where the proteins are first taken to the periplasm and then folded before becoming secreted through large channels formed in the outer membrane by proteins known as secretins [123]. This secretion system is, however, absent in *B. pertussis*, *B. parapertussis* and *B. bronchiseptica*, but present in *B. avium* [126]. T3SS is a complex export structure delivering the effector proteins directly from the bacterial cytoplasm into the host cell cytosol and thus altering the function of the host cell. The gene encoding T3SS has been identified in Bordetellae [127], but the protein expression of T3SS in

B. pertussis was only recently demostreted [128]. It was shown that a functional T3SS is expressed by the clinical B. pertussis isolates but not by the laboratory strains suggesting that the long-term laboratory culture has significantly changed the gene expression and the protein production. T3SS was also shown to participate in the bacterial colonisation and survival in the host by targeting the innate immune system. The role of T3SS in the host-pathogen interaction has been widely studied among species other than Bordetellae [129]. Both in animal models and in human infection, T3SS activity is well correlated with the infection progression and outcome [130]. Thus, the recent findings of the functionally active T3SS in B. pertussis by Fennelly and colleagues [131] will improve our understanding of the immune responses to B. pertussis infection and especially to this important immunomodulator and virulence factor. [132]

PTX is secreted via T4SS named as pertussis toxin liberation (Ptl) composed of nine proteins (figure 5) [133]. PTX is assembled from the subunits in the periplasmic space, and the Ptl proteins are likely to form a large complex spanning both the inner and outer membranes. It seems that the Ptl T4SS mediates the secretion of PTX to the extracellular space, but is

not involved in the host cell translocation of PTX. [134]T5SS is the simplest and the most common secretion systems. Two Sec-dependent T5SS pathways are known: the autotransporter (AT) pathway and the two-partner secretion (TPS) pathway. In the AT system, the protein itself mediates the secretion across the outer membrane. Among the Bordetella AT proteins, Prn is the most widely studied, and in fact, it has been proposed as the archetype of the AT proteins of all bacteria [135]. In the TPS, an additional pore-forming transporter protein is required for the secretion. A Bordetella virulence factor, FHA, is used as a generic model for the TPS pathway [136]. [137, 138] T6SS is the most newly described secretion system, and it has been proposed to form an apparatus that punctures the target cell membranes delivering the bacterial effector proteins directly to the target cell cytosol [139]. The role of T6SS in B. pertussis is not yet known, but the T6SS gene clusters were recently found in B. bronchiseptica and B. parapertussis [140].

BORDETELLA PERTUSSIS IS CHANGING

Changes to circulating *B. pertussis* strains seem to be one of the suggested causes of the pertussis cases increase. Indeed, the genome of the current circulating bacteria differs from the isolated ones used in the manufacture of vaccine component (table 1) [27] and in particular, it has been shown that modification occurred in pertussis toxin (Ptx) and in pertactin (Prn), which elicit protective immunity and are two of the used components of the ACV [92]. Other modifications have been shown in the remaining *B.pertussis* proteines, which are included in ACVs: the filamentous hemagglutinin (FHA); and serotype 2 and 3 fimbriae (Fim2 and Fim3) [93].

Table 1

Antigenic alleles in current acellular pertussis vaccines compared with current circulating *B pertussis* strains

Vaccine Strains	Circulating Strains
prn1-ptxA2-ptxP1-fim3A	*prn2-ptxA1-ptxP3-fim3A/B*
prn1-ptxA4-ptxP1-fim3A	_

Ptx is composed of five subunits encoded by contiguous cistrons within a single polycistronic operon: one of the them is PtxA, which shows catalytic and toxic activity. Of the five subunits, PtxA is the most immunogenic and also the most polymorphic, indeed it has eight allelic variants of which ptxA2 and ptxA4 are present in strains used to produce many acellular vaccines, including those used in the United States and Europe. While in several European countries the most common ptxA allele is a nonvaccine type allele, ptxA1, which, together with ptxA3, ptxA6 and ptxA7 codes for an identical protein, PtxA1. This protein and the PtxA in the strains used for the vaccine development have one or two differences in the amino acids constituting Band T-cell epitopes (figure 6). These observations provide strong evidence that variation observed in PtxA is immunologically relevant [92] [94]. The production of most of the different virulence factors is under the control of a two-component signal-transduction regulatory system named BvgAS. [95]

[96]. Finally, the nucleotide sequences of the four most common variants are presented in figure 7.

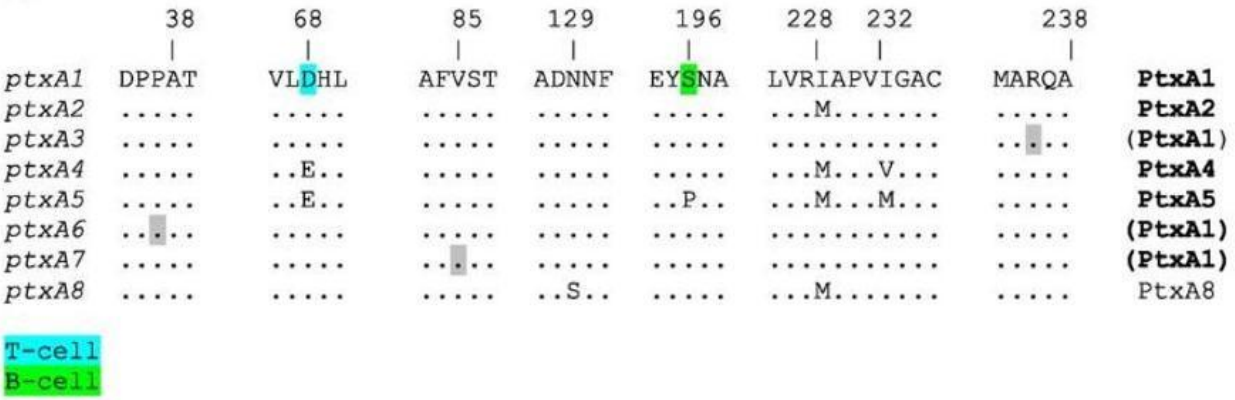


Figure 6. Pertussis toxin subunit A variants found in *B. pertussis* populations. Polymorphisms in PtxA known to affect T-cell and B-cell epitopes are highlighted in blue and green, respectively. Source [28].

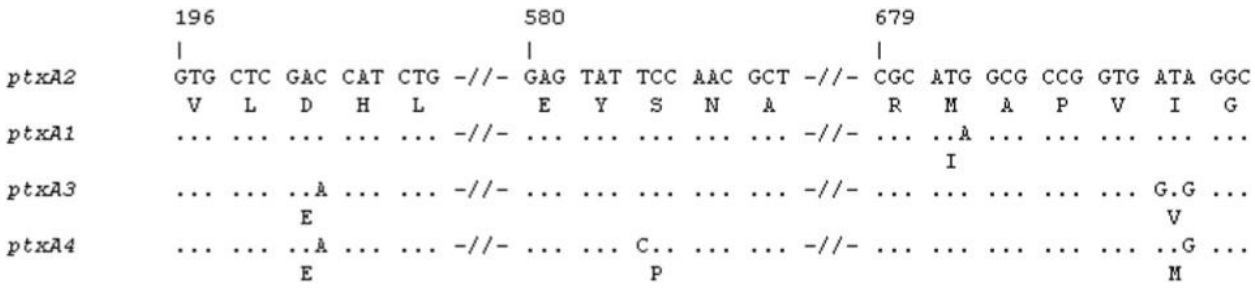


Figure 7. The nucleotide sequences of the four most common Pertussis toxin S1 subunit variants.

For most bacterial virulence control systems, BvgAS has generally been considered as a biphasic transition mediator. In the Bvg+ phase, BvgAS is active, and Bvgactivated virulence genes are expressed. Inactivation of BvgAS by the presence of modulating signals or by mutation results in expression of the Bvg− phase, characterized by the expression of Bvg-repressed genes and lack of expression of Bvgactivated loci. The B. pertussis Bvg− phase is relatively uncharacterized, however, the Bvg− phase of B. bronchiseptica allows survival under conditions of severe nutrient deprivation. These and other results have led to the

hypothesis that BvgAS functions as a molecular switch, inducing expression of the Bvg+ or Bvg− phases in response to whether the bacteria are located within or outside the mammalian respiratory tract respectively [97]. Between the toxins regulated by BvgAS system there is also Ptx; BvgS is an inner-membrane- spanning protein expressing multiple kinase activities. In the active state, it activates BvgA via phosphorylation. Phosphorylated BvgA, a cytoplasmic transcriptional regulator, then binds to the operator sites of the ptx gene promoter region, where it interacts with the RNA polymerase and activates transcription [96].

But also the *B. pertussis* toxins promoter (ptxP) has multiple alleles of which ptxP1, ptxP2, and ptxP3 are predominant worldwide (figure 8). The ptxP SNPs found in ptxP3 were in a DNA segment known to bind BvgA, given that it is not surprising that ptxP3 is associated with changing in Ptx expression. In particular, ptxP3 *B. pertussis* strains have 1.6 times greater production of pertussis toxin compared with ptxP1 ones, suggesting increased fitness related to the mutation [94]. Increased toxin production may exert an advantage in vaccinated hosts by delaying an effective immune response, thus allowing for enhanced transmission. [92].

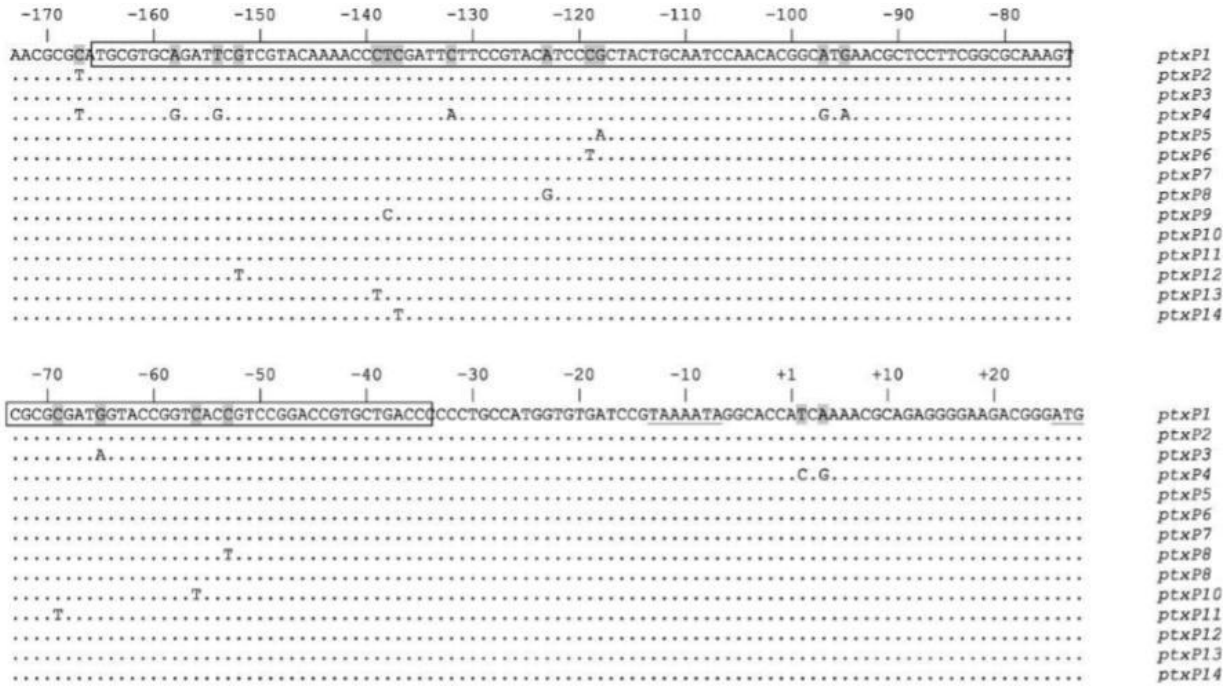


Figure 8. Alleles of pertussis toxin promoter (ptxP) observed worldwide. Bases are

numbered – 173 to +27 relative to the start of transcription (+1). The region to which BvgA bind is underlined. Source [28].

Consequently, is not a surprise that, a study in the Netherlands correlated the temporal epidemiologic increase in ptxP3 to increased incidences of pertussis hospitalization and deaths [98].

Pertactin is a protein that helps pertussis bacteria attach to the lining of the airways, and the gene that produces it has been described in thirteen alleles variants. Most vaccine strains contain the prn1 or prn7 alleles and just the Swedish vaccine strain harbors prn10. The allele variation is mainly generated by two repeat regions, although SNPs are also observed. Region 1 contains repeats comprised of five amino acids and is located proximal to the RGD motif (arginyl-glycyl- aspartic acid-motif) implicated in binding to host receptors. The second region contains PQP repeats (figure 9, 10). The two repeat regions are immunodominant and induced protective antibodies in a mouse model. In the late 1970s, two novel prn alleles, prn2 and prn3, replaced the vaccine-type prn1 which, until the introduction of vaccination, was by far the most predominant type [94]. Nowadays, prn2 genotype is the most common one [28], but *B. pertussis* strains without Prn are becoming more and more common. However, at present, it seems that prn-deficient strains do not differ from the prn-producing strains in terms of the clinical disease they cause [99].

In conclusion, PtxA and Prn confer immunity in humans and therefore non-vaccine types of these antigens are expected to increase strain fitness in vaccinated populations [94]. Moreover, changes in regulatory region as ptxP can also facilitate *B. pertussis* survival in our body.

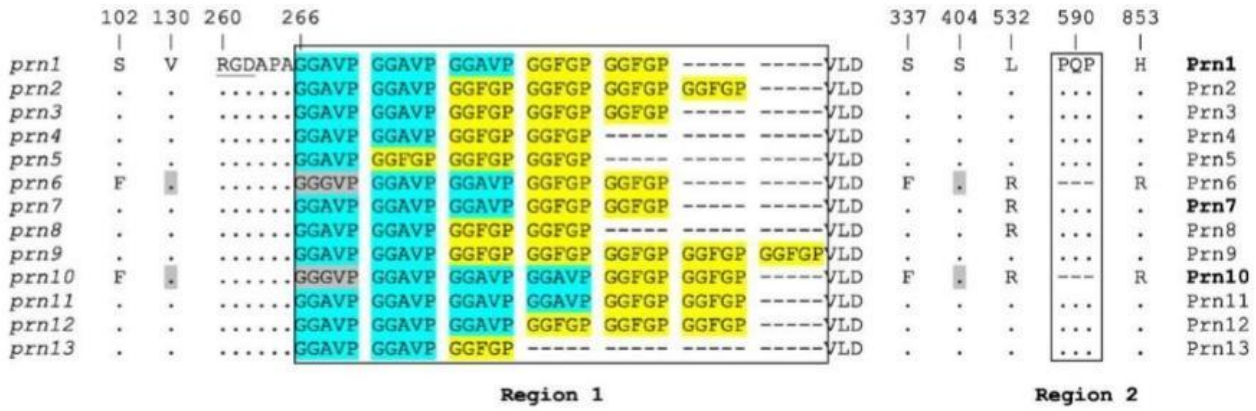


Figure 9. Pertactin variants found in *B. pertussis* populations. Allele and protein designations are shown on the left and right. Dots and dashes indicate identical and absent amino acids, respectively. Positions with silent mutations are shaded. Numbering is relative to the N-terminal methionine of Prn1. The two regions (1 and 2) with five and three amino acid repeats, respectively, have been blocked. The five amino acid repeats occur as three variants which have been highlighted. Source [94].

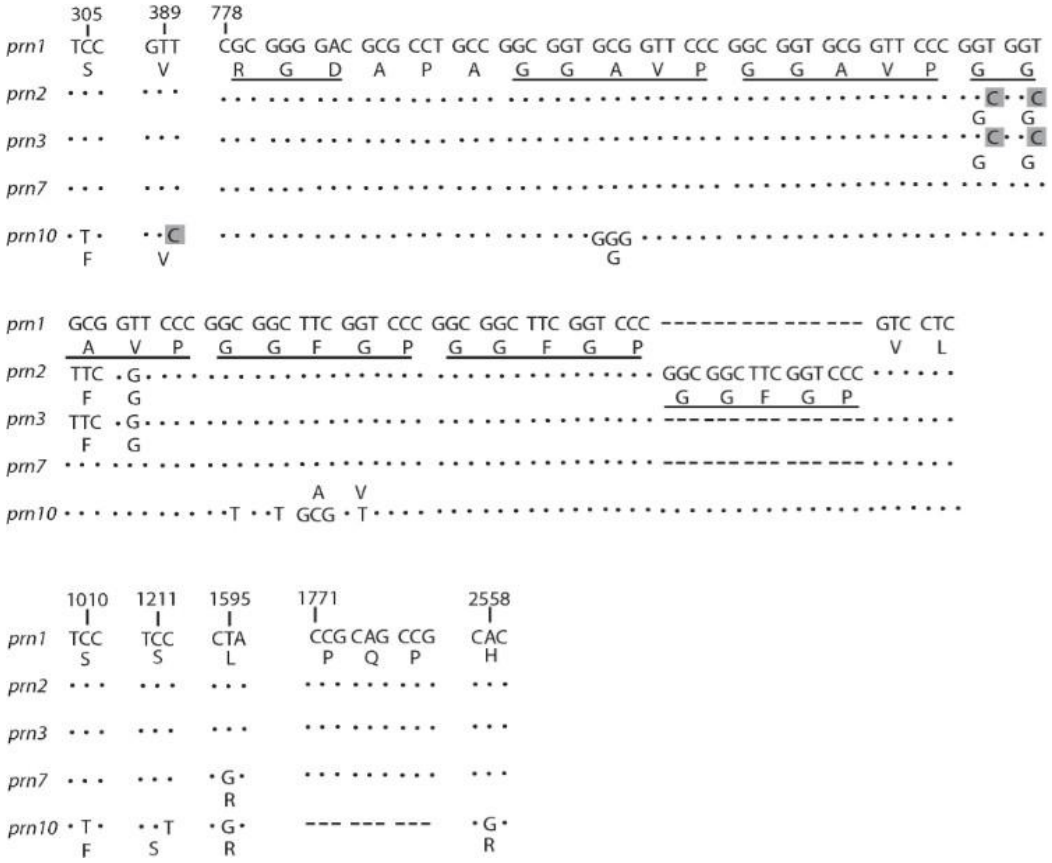


Figure 10. Variants of Prn observed in the *B.pertussis* population. Depicted are the DNA sequence and corrisponding amino acid sequence of the single polymorphic regione observed. The RGD motif implicated in binding to host receptors has been underlined. Repeat motifs has been underlined beside. Dots and dashes indicate sequence identity with Prn1 and gaps, respectively.

The gene encoding fimbriae is the other virulence gene which is also subject to variation due to mutation. *B. pertussis* strains contain Fim2 and/or Fim3 fimbriae serotypes. Two and four alleles of fim2 and fim3 genes have been identified up to now [102] (figure 11).

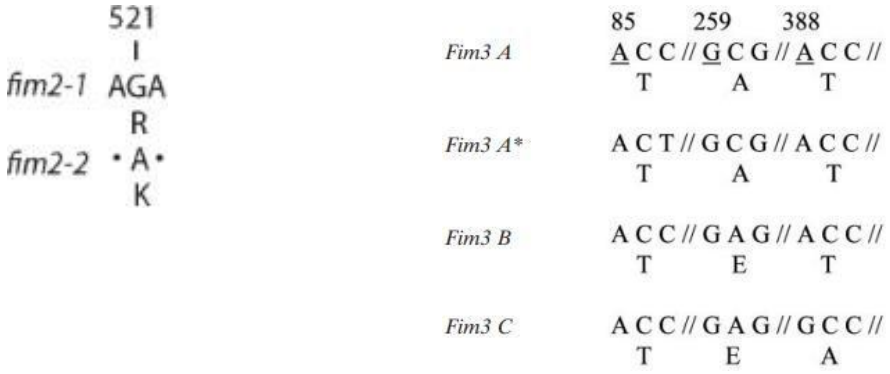


Figure 11. Polymorphic regions in the *B.pertussis* fimbria genes (fim2 and fim3). The nomenclature of the fim3 variants corrispond to: fim3A to Fim 3-1, Fim3 A* to Fim 3-4, Fim 3B to Fim 3-2, Fim 3C to Fim 3-3.

FHA is a dominant adhesion molecule of *B. pertussis* against eukaryotic cells [103, 104]. It is also highly immunogenic and that is why it is included as an antigen in many of the currently used acellular vaccines. In 2002, in a study performed in The Netherlands, 196 strains from widely separated geographic regions (The Netherlands, Filand, Italy, Japan and the United States) were investigated. The fhaB gene from 13 strains was sequenced, between bases 1 and 6612. This is the region that codes for the secreted, processed molecule incorporated in ACVs. Subsequently, sequencing of fhaB was confined to the region comprinsing bases 2250 to 2750, which contained the single polymorphic site identified (which contain the polymorphic locus). Only a single nonsilent mutation was observed in fhaB [93] (figure 12).

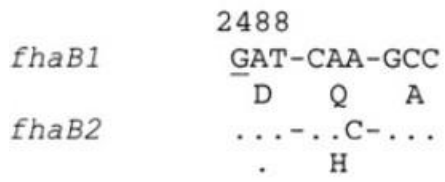


Figure 12. Alleles of gene coding for fhaB protein. Number refer to undelined residues. Dots indicate indentity, and codons are separeted by dashes.

Given the infection increase in Italy described by Palazzo et al. as well as other Authors, we investigated B. pertussis strains circulating in Italy.

DIAGNOSIS

Diagnosis of pertussis is based on clinical suspicion combined with proper laboratory tests. The World Health Organization and European Center for Diseases Prevention and Control (ECDC) provide the case definition for pertussis suspicion [141,142]. According to ECDC's and WHO's clinical case definition, a suspicion of pertussis should be considered when a patient has a cough of two weeks or longer and at least one of the following symptoms: 1) paroxysms of cough

2) inspiratory whooping or 3) post-tussive vomiting. A laboratory confirmed case should have at least one of the following: isolated *B. pertussis* from a clinical specimen, detection of *B. pertussis* nucleic acid from a clinical specimen or *B. pertussis* specific antibodies. Diagnostic laboratory test should be selected based on the age of the patient and the duration of the symptoms. Commonly used diagnostic methods are those listed in the WHO's case definition; culture, polymerase chain reaction (PCR) and serology. Culture is thought to be the golden standard for the diagnosis, since its specificity is known to be high. However, the sensitivity of the culture is affected by several factors. The positivity rate of the culture decreases drastically if the sample is taken more than two weeks after the beginning of the symptoms or if the patient has received antimicrobial treatment. The sensitivity falls to 1-3%, if the specimen is collected after three weeks from the onset of cough [143]. The sensitivity is reduced also if the specimen is improper, meaning that it is a per nasal or throat swab or a sweep from the anterior nares [144]. The correct place for the specimen collection is in the posterior nasopharynx as *B. pertussis* mainly attaches to the ciliated epithelium of the nasopharynx [145]. In addition to swabs, nasopharyngeal aspirates are often taken, especially from infants. The specific medium for *B. pertussis* culture is Reagan-Lowe agar and Bordet-Gengou agar. Proper culture condition is ambient ≥+35°C and additional CO2 is not needed [146]. Colonies of light grey, highly convex, pearly and mercury-like appearance are visible in average 72 hours but longer incubation increases the sensitivity [147]. The clinical laboratories usually provide the positive result after three days, but the confirmation of the negative result is given after seven days of incubation [148].

Besides of the diagnostic purpose, collecting the *B. pertussis* strains from pertussis patients

is important for molecular typing of the circulating bacteria and antibiotic susceptibility testing. Molecular typing helps to understand the bacterial adaptation to vaccine induced immunity and the emergence of new variants of *B. pertussis*. Antibiotic susceptibility testing is important to monitor antimicrobial resistance strains. For example, recently, erythromycin resistant *B. pertussis* has emerged in China and Iran [149, 150]. Amplification of the nucleic acid using PCR has sustained its position as a valuable diagnostic method since the early 1990's, when it was described as a sensitive method to detect *B. pertussis* from the nasopharyngeal specimens [151, 152]. The benefits of PCR are its speed, sensitivity and specificity. In the PCR reaction, nucleic acid is amplified, allowing to detect even very low numbers of bacteria or non-viable bacteria. The sensitivity of PCR compared with culture can be fourfold higher and it can be used in samples taken from patients with longer period after onset of the symptoms [153]. This is a clear advantage for pertussis diagnostics, as a positive result can be detected even after the culture becomes negative [154]. *B. pertussis* genome contains several elements which have been used as a target in the diagnostic PCR, such as the commonly used pertussis toxin (PTX) promoter [155] and insertion sequence (IS) element 481 [156]. The most widely used target is the transposable element IS481, which is present in more than 200 copies in the B. pertussis genome [157]. However, IS481 sequence is also found in the genome of *B. holmesii*, but only in 8-10 copies [158]. Due to this cross reactivity of IS481 between the two species, secondary targets such as RecA have been proposed to differentiate them[159, 160]. However, this may decrease the analytical sensitivity of the PCR [161] since there is only one copy of this gene in the *B. pertussis* genome. The high number of IS481 copies increases the sensitivity of the PCR which may increase the risk for false positive results mainly due to contaminations at different stages of the process [162].

After infection, increase is noticed in the serum concentration of immunoglobulins (Ig) G, IgA and IgM against specific antigens of *B. pertussis*, such as pertussis toxin (PTX), pertactin (PRN) and filamentous hemagglutinin (FHA). The basis of serology is the measurement of antibody concentrations against *B. pertussis* specific antigens from serum samples using enzyme-linked immunosorbent assay (ELISA). Purified PTX and FHA are commonly used as a capture molecule, PRN and fimbriae (fim) less extensively [163]. Among these antigens, only PTX is specific for *B. pertussis* and it is thus recommended to be used in serological diagnosis [163, 164]. The first serological pertussis ELISA described for the diagnostics had

sonicated whole bacteria as a coating molecule and this method is still used in pertussis diagnostics in Finland [165]. Increase in the IgG concentration against PTX, FHA and PRN is seen after infection. PTX is *B. pertussis* specific whereas anti-FHA and anti-PRN IgG may be caused by cross-reactivity with other Bordetellas or other species such as *Haemophilus* species and *Mycoplasma pneumoniae* [163]. IgA against PTX, FHA and PRN are produced less extensively, only in 20 – 50 % of the subjects [166]. Even if PTX is specific for B. pertussis, serological tests do not distinguish the antibodies produced after vaccination or during *B.pertussis* infection, as it is present both in the vaccine and in the bacteria. This may interfer with interpretation of the results and that is why vaccination status and age of the patient should be taken into accout when evaluating the results. Increase in the antibody concentration is seen rather late, only after one or two week from the onset of the symptoms. Consequently, serology can be performed from paired sera, the first taken from the acute phase and the second from the convalescent phase. However, in the clinical practice a single serum is used more commonly. In neonates and infants younger than two years of age, PCR or/and culture should be preffered as a diagnostic method, due to the possible interference of vaccination on antibodies production. In addition, the antibody production after infection may be slow in this age group [163,166]. In older children, adolescents and adults serology is the most commonly used method in the diagnostics. table 2 summarizes the three commonly used pertussis diagnostic methods.

Method	Duration of symptoms	Suitable age	Advantages	Disadvantages
Culture	< 3 weeks	All patients	Cheap Collection of circulating strains for surveillance High sensitivity in infants	Decreased sensitivity with time Time-consuming
PCR	up to 4 – 6 weeks	All patients	Sensitive Specific Speed Longer usability	Contamination risk Specificity depends on the target
Serology	2 – 12 weeks	>2 years of age >1 year from the last pertussis vaccine	Longer usability Easy sampling	No differentiation between vaccine and infection induced response

Table 2. Summary of culture, PCR and serology in pertussis diagnostics.

PERTUSSIS VACCINATION

After the first isolation of *B. pertussis* in the early 1900s, and due to the high mortality of whooping cough, research on vaccine development against this serious disease was started. The first experimental vaccine against pertussis was designed in 1933. Slight protection was reported with this vaccine composed of suspended bacteria. By that time, the same preparation was used as a cure for pertussis infection and to immunize against pertussis [194]. Between the 1940s and the 1950s several industrialized countries started the large-scale vaccinations against pertussis. Until this milestone, pertussis had been the main cause of infant mortality and more than every other child had suffered from it before school entry [195]. The introduction in 1950s of the whole cell pertussis vaccine (WPV) decreased drastically the incidence and mortality from pertussis.

VACCINE TYPES

Two types of pertussis vaccines are current in use; whole cell pertussis (DTwP) and acellular pertussis (DTaP) vaccine, both in combination with diphtheria (D) and tetanus (T). Recently Haemophilus influenzae type b (Hib), hepatitis B (HepB) and inactivated poliovirus (IPV) vaccines have also been included in the combination vaccine. For the vaccine nomenclature, the size of the letter describes the amount of antigen included in the vaccine. Capital letter indicates higher amount of antigen whereas small letter is a sign of reduced antigen composition, which is common for booster vaccines.

At the beginning of vaccinations, WPV was administrated as a monocomponent, but soon a combination of DTwP was available and recommended [6]. The isolation of pertussis toxin and its use as a vaccine component was not similarly possible as it was with diphtheria and tetanus toxoids, because of the difficulties in growing of *B. pertussis*. As a consequence, whole-cell vaccine is produced from the heat-killed bacteria. The bacterial strains selected for the synthesis of the vaccine were those circulating during the development phase of DTwP [196]. However, the reproducibility of the DTwP vaccine is not simple because of the difficulty in growing *B. pertussis*. The effect of DTwP vaccination on pertussis incidence was significant. In fifteen years, the incidence of pertussis in the USA decreased from 209 cases/100,000 to 51 cases/100,000 [197]. Even DTwP was effective against pertussis; the drawback of this vaccine was its reactogenicity. This aspect is extensively described in a review by Mattoo and Cherry [6]. Local reactions at the injection site such as redness and swelling were commonly recorded, as well as fever (≥38°C). The most adverse reactions were related to neurological disorders and sudden deaths, although they occurred rarely. The fear of the adverse vaccine reactions even led to the discontinuation of pertussis vaccination in Sweden from 1979 to 1996 [198]. In Japan, two fatalities led to a temporary, two months, suspension of vaccination. A broad refusal of the DTwP vaccine was followed, causing the vaccine coverage to decline to 10% [199]. Nevertheless, the DTwP vaccines are still administrated in Poland and Serbia in Europe and in most of the developing countries as the cost of DTwP vaccine compared with DTaP is more than 10-times lower, which makes DTaP not affordable in low-income countries [200, 201, 202]. The development of a replacement vaccine for DTwP started after the 1970's, when it was technically possible to extract and purify the antigen components of *B. pertussis* [6]. Acellular vaccine is composed of the purified antigens of *B. pertussis*. Japan was the first country to report the

replacement of DTwP with DTaP in 1981 [199]. After that, most of the developed countries have followed. In the 1990's series of randomized, double-blinded efficacy trials were conducted to evaluate the immunogenicity, efficacy and safety of acellular vaccine. These studies were performed in countries such as Italy, Sweden and Germany, who all had previously used DTwP in their vaccine programs [203, 204, 205]. The supportive data obtained on DTaP vaccines safety and efficacy led to the licensure of the vaccine in many developed countries. Currently available DTaP vaccines contain one to five antigens: PTX, PRN, FHA, Fim2 and Fim3 in combination of PTX-FHA, PTX-PRN-FHA, PTX alone or all five antigens together. To strengthen the immune response, antigens are combined with alum adjuvant. DTaP is significantly less reactogenic and more tolerated compared with DTwP and due to this DTaP can also be administrated to adolescents and adults [206, 207].

The recent large pertussis epidemics in different parts of the USA and in Australia have demonstrated a new pattern of infection. High incidence has been noticed in pre-adolescents and young adolescents who have received their primary pertussis vaccines during the transition from DTwP to DTaP. These age- groups are the first cohorts that have received purely acellular pertussis vaccines as a primary vaccine. Several retrospective studies have been conducted to estimate whether the transition of vaccine has affected the incidences rates. Several recent publications have shown that widely used DTaP vaccines seem to provide a shorter period of protective immunity compared with DTwP [191, 202, 217, 218].

DIFFERENCES IN IMMUNITY ACQUIRED BY INFECTION AND VACCINATION

Infection with *B. pertussis* or immunisation with WPV or ACV induce the production of specific antibodies within a few weeks after the exposure to bacteria or vaccination. The protective immunity to pertussis after natural infection or vaccination is not lifelong; the values of antibodies decrease over time until re-infection or re-immunisation induces the secondary immune response. However, the duration and the mechanism seem to differ between the immunity induced by the infection and vaccination, and further, between the WPV and ACV vaccination [208]. The duration of pertussis immunity has been extensively studied alongside the vaccine development. As the research data was reviewed, it was shown that the estimates the duration of the infection-induced and vaccination-induced immunity were from 4 to 20 and 4 to 12 years, respectively, and the protective immunity after the vaccination with aP wanes faster than after vaccination with wP [208]. However, it seems that the protective immunity against the disease lasts longer than against the infection, and further, the symptomatic or asymptomatic re-infection may serve as a booster for immunity [6, 208, 209]. The mechanisms of the immunity acquired by infection and vaccination are different. The natural infection induces IgA, IgG and IgM responses to specific antigens and the whole bacteria whereas the vaccination induces only the production of IgG and IgM antibodies. IgA has been shown to facilitate the binding, phagocytosis, and killing of B. pertussis and to induce cellular immune functions [210]. Thus, the important and versatile role of IgA may explain some of the differences between the immunity acquired after the natural infection and vaccination. Further antibodies are only produced against the antigens included in the vaccine when ACV is used. Differences in the cell-mediated immunity have also been shown. After the natural infection and WPV vaccination, T-helper 1 cells (Th1) are activated, whereas T-helper 2 (Th2) biased cells or both Th1 and Th2 cells are generated after ACV vaccination [211]. Th cells are involved in activating and directing other immune cells to the infection site. In a simplified manner, Th1 cells are evoked by bacterial antigens and they activate the phagocytic immune cells to kill the bacteria, whereas Th2 cells trigger the antibody production by B cells via the production of interleukins [6, 208, 209, 210, 212, 104].

HERD IMMUNITY

It is well described that in a population where an effective vaccine against any disease is used with high coverage, even the unimmunised members are less likely to encounter the disease. Due to the mass vaccination, the risk of transmission is reduced and thus the unimmunised and imcompletely immunised members as well as the vaccine failures are somewhat safe from the disease [213]. When the herd immunity increases due to vaccination with high coverage, the circulation of the corresponding agent may cease [214]. Herd immunity is an advantage of extensive vaccination and plays a significant role in the control and prevention of pertussis [213]. Thus, herd immunity can be exploited on the design of vaccines and vaccination schedules even though the immunity against pertussis is not yet fully understood. However, the protective effect of herd immunity is lost if vaccination coverage decreases due to anti-vaccine movements and parental refusal of vaccinating their children. Due to the long history of extensive and effective vaccinations, many young parents have never confronted pertussis and they may thus underestimate the value of vaccination. This again poses an unnecessary threat of pertussis to their children, to their children contacts, and further, to the community, due to the riduced herd immunity [215, 216].

PREVENTING EARLY INFANT MORTALITY: PROPOSED STRATEGIES

Developing a new vaccine against pertussis will take several years before it can be made available. Meanwhile, a more optimal use of the current vaccines should be made. What matters is to protect the infants too young to be vaccinated that are those for whom the disease can be more dangerous. In order to achieve this goal numerous strategies have been proposed in literature.

Booster in adolescents and adults

In some countries adolescents and adults are offered boosters of aP vaccine in combination with tetanus toxoid and reduced-dose diphtheria vaccine. Although these programmes have an impact in the directly targeted populations, there is as yet no statistically significant evidence that they have had a significant impact on incidence of severe pertussis in infants [1, 220].

Neonatal pertussis vaccination

In view of data demonstrating protection against severe pertussis disease in infants after a single dose, the immunization of infants at birth has been considered as a strategy potentially worth pursuing. Despite the birth dose of DTaP is well tolerated, compared with the control group, infants who received the vaccine at birth demonstrated a significantly lower response to diphtheria toxoid and to 3 of 4 pertussis antigens when measured at 7 months [219].

Vaccination of health-care workers

In many countries, vaccination of health-care workers with acellular pertussis containing vaccines is recommended and several studies have shown that transmission in health-care settings poses substantial risk of infection for infants and immunocompromised individuals. However, no direct evidence of this strategy's efficacy on the reduction of morbidity and mortality in neonates was obtained. Vaccination of health care workers is therefore considered only partially effective in preventing nosocomial spread of infection [1, 220].

Cocooning strategy

The cocooning strategy, which has been recommended in certain developed countries, refers to the approach in which those who are likely to be the source of infection for the unimmunized infants - caregivers, family members - are vaccinated. Even if cocooning may have an impact in reducing the prevalence of pertussis in infants, costs and logistical barriers to widespread implementation of this strategy were major limitations and raise doubt as to whether cocooning is the best overall approach to reduce the burden of pertussis in young children. Moreover, Rivero Santana et al. showed that vaccination of pregnant women was more effective than cocooning [220].

Vaccination of the expectant mother during pregnancy

Vaccination of women during pregnancy with an adult formulation of acellular pertussis vaccine in combination with tetanus and diphtheria toxoids is recommended in several industrialized countries to boost the levels of maternal antibodies that are transferred transplacentally and protect infants during the period of life when they are more likely to succumb to pertussis [98]. Evidence consistently indicates that maternal immunization with acellular pertussis containing vaccine during the third trimester of pregnancy is safe and highly effective in protecting infants from pertussis [1]. This outcome is probably primarily due to the direct protection conferred by the transfer of maternal antibodies, with some contribution from reduced risk of transmission through reduced likelihood of peripartum pertussis in the mother. Although concern exists regarding the interference of transplacentally derived antibodies with the infant's immune response to their subsequent recommended infant DTaP vaccinations, it has been shown that infant immune responses to acellular pertussis vaccines were not affected by pre-existing antibodies against PTX, but interference was seen with the whole-cell pertussis vaccine [221].

AVAILABLE VACCINES AND NATIONAL IMMUNIZATION PREVENTION PLAN 2017-2019

Several combined acellular pertussis vaccines are available to protect children, adolescents and adults in Italy:

- DTPa (diphteria, tetanus, acellular pertussis trivalent): Infanrix DTPa (GlaxoSmithKline, suspension for injection available in 0.5-mL);
- dTpa (diphteria, tetanus, acellular pertussis trivalent to protect adolescents and adults): Boostrix (GlaxoSmithKline, suspension for injection available in 0.5-mL), contains reduced doses of diphtheria anatoxin and pertussis antigens and normal dose of tetanus anatoxin; [167]
- DTPa/IPV (diphteria, tetanus, acellular pertussis, inactiveted poliomyelitis tetravalent): Polioinfanrix (GlaxoSmithKline, suspension for injection available in 0.5-mL): Tetravac (Sanofi Pasteur MSD, suspension for injection available in 0.5-mL);
- DTPa/IPV/Hib (diphteria, tetanus, acellular pertussis, inactiveted poliomyelitis, *Haemophilus influenzae* type b pentavalent): Pentavac (Sanofi Pasteur MSD, suspension for injection available in 0.5-mL);
- DTPa/IPV/Hib/HB (diphteria, tetanus, acellular pertussis, inactiveted poliomyelitis, *Haemophilus influenzae* type b, hepatitis B hexavalent): Infanrix Hexa (GlaxoSmithKline, vial+ suspension for injection available in 0.5-mL).

In the last years, two new hexavalent vaccines have been licensed and commercialized worldwide [168]: Hexyon and Vaxelis. Their composition is summarized in table 3 [169, 170, 171]

Combination	Commercial name	Company	DT	TT	PTX	FHA	PRN	FIM	HBs	PRP-T PRP-OMPC	Polio type 1/2/3	Adjuvants
DTPa-HB- IPV-Hib (liquid)	Vaxelis®	Sanofi Pasteur and MSD	20 IU	40 IU	20 mcg	20 mcg	3 mcg	5 mcg	10 mcg	3 mcg/50 mcg**	40 D/8 D/32 D	Aluminium phosphate Aluminium hydroxyphosphate sulfate
	Hexyon®	Sanofi Pasteur	20 IU	40 IU	25 mcg	25 mcg	–	–	10 mcg	12 mcg/2–36 mcg*		Aluminim hydroxide, hydrated
DTPa-HB- IPV + Hib (lyophilized)	Infanrix® Hexa	GSK	30 IU	40 IU	25 mcg	25 mcg	8 mcg	–	10 mcg	10 mcg/25 mcg*		Aluminium hydroxide, hydrated, Aluminium phosphate

DT: Diphtheria toxoid, TT: Tetanus Toxoid, B. pertussis antigens -> (PTX: Pertussis Toxoid, FHA: Filamentous Haemagglutinin, PRN: Pertactin, FIM: Fimbriae type 2 and 3), HBs: Hepatitis B surface antigen, Hib Antigens -> (PRP-T: Polyribosylribitol phosphate conjugated to tetanus toxoid, PRP- OMPC Polyribosylribitol phosphate conjugated to meningococcal protein), Polio type 1: Poliovirus (Inactivated) Type 1 (Mahoney), Polio type 2: Poliovirus (Inactivated), Type 2 (MEF-1), Polio type 3: Poliovirus (Inactivated) Type 3 (Saukett).

*Uses PRP-T as Hib Antigen.

**Uses PRP-OMPC as Hib Antigen.

Table 3. Hexavalent combination vaccines. Summary of characteristics of main hexavalent vaccines available in Europe.

The Italian Immunization schedule includes a cycle of three hexavalent vaccinations for children: at the 3rd month (61st day of life), 5th month and 11th-13th month of life.

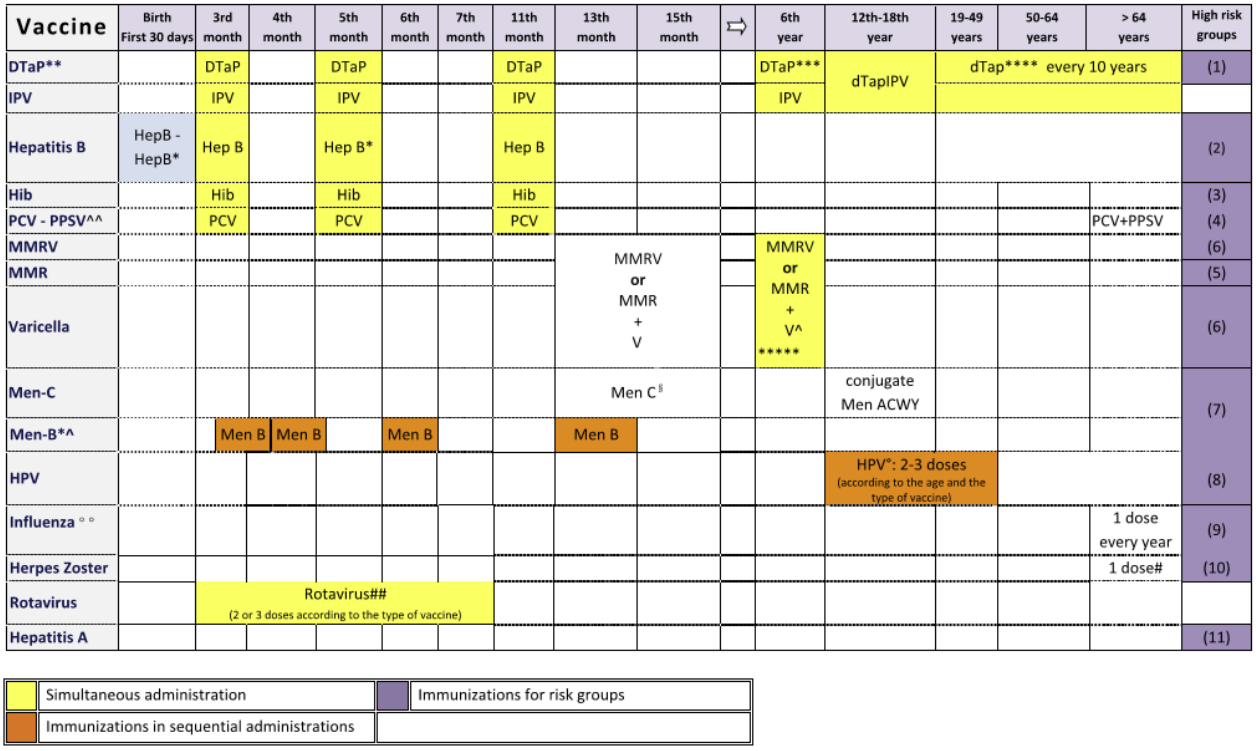
A booster dose for DTPa / IPV is expected at 5-6 years of age and a second dose at 14-15 years (range 11-18) for dTpa.

dTpa is reccomended every 10 years from age 19, and also for pregnant women in the third trimester (ideally 28 weeks).

The Italian National Immunization Prevention Plan (PNPV) identifies vaccines actively offered free of charge to target populations within the National Health Service.

The National Plan for Vaccine Prevention 2017-2019 has expanded the vaccination offer including new vaccines, enlarging the target population and introducing for the first time in Italy a life- course approach to vaccination. A “lifetime immunization schedule” is aimed at reducing the burden and the related costs of vaccine-preventable diseases through effective vaccination programs. However, to counteract the national steady downward trend in the uptake of vaccinations that caused a drop of the vaccination coverage below the 95% threshold to allow herd immunity, it was decided to make 10 vaccinations mandatory by the law 119/2017. In particular, in addition to already mandatory vaccinations against diphtheria, tetanus, hepatitis B and poliomyelitis, those against measles, mumps, rubella (MMR), varicella, pertussis and Haemophilus influenzae type b (Hib) were added to the list. According to the law, all unvaccinated children cannot attend preschool services until the age of 6 years and fines (from 100 to 500 Euros) are provided for parents. Moreover, this law provided, in its first application, a catch-up campaign for children up to the age of 16 years and the free-of-charge offer of all mandatory and recommended vaccines to each child not yet vaccinated according to the previous NPVP. The NPVP includes also several at risk categories, such as pregnant women, healthcare workers and subjects suffering from chronic diseases, to whom specific vaccinations, free of charge, are offered. The vaccinations of pregnant women have different purposes. In order to decrease the pertussis risk in newborns in the first months of life, a booster immunization of DTPa is recommended, at every pregnancy, between week 27th and 36th. Instead, the influenza vaccine administration to pregnant women during the second or third quarter is mainly aimed at avoiding the risk of serious disease complications for both the mother and the fetus. Another group of at risk subjects included in the NPVP is that

made up by healthcare workers. According to the plan, “an adequate immunization of the healthcare workers is essential for the prevention and control of infections (anti-hepatitis B, anti-influenza, anti-measles-mumps-rubella, anti-varicella, anti-pertussis)”. Finally, almost all the vaccinations foreseen by the NPVP are offered free of charge to subjects suffering from specific diseases, including cardiovascular, respiratory, hepatic, renal, neoplastic and metabolic disorders, in addition to immunosuppression that exposes them to an increased risk of contracting invasive infectious diseases [172]. The current suggested vaccination schedule in Italy is illustrated in figure 13. [173]



Vaccine	Birth First 30 days	3rd month	4th month	5th month	6th month	7th month	11th month	13th month	15th month	⇨	6th year	12th-18th year	19-49 years	50-64 years	> 64 years	High risk groups
DTaP**		DTaP		DTaP			DTaP				DTaP***	dTapIPV	dTap**** every 10 years			(1)
IPV		IPV		IPV			IPV				IPV					
Hepatitis B	HepB - HepB*	Hep B		Hep B*			Hep B									(2)
Hib		Hib		Hib			Hib									(3)
PCV - PPSV^^		PCV		PCV			PCV								PCV+PPSV	(4)
MMRV								MMRV or MMR + V			MMRV or MMR + V^ *****					(6)
MMR																(5)
Varicella																(6)
Men-C								Men C§				conjugate Men ACWY				(7)
Men-B*^		Men B	Men B		Men B			Men B								
HPV												HPV°: 2-3 doses (according to the age and the type of vaccine)				(8)
Influenza ◦◦															1 dose every year	(9)
Herpes Zoster															1 dose#	(10)
Rotavirus		Rotavirus## (2 or 3 doses according to the type of vaccine)														
Hepatitis A																(11)

	Simultaneous administration		Immunizations for risk groups
	Immunizations in sequential administrations		

Figure 13 Immunization schedule included in the Italian National Immunization Plan (PNPV), 2017–19. *For children born from HBsAg positive mothers. It's a four-dose schedule: the 1st dose within the first 12–24 h of life concurrently with specific Hepatitis B immunoglobulins; the 2nd dose after 4 weeks, 3rd dose following the lifetime immunization schedule, after the 60th day of life, in co-administration with the hexavalent vaccine. *^Even leaving the final decision to the local administration regarding the best possible schedule according to the local vaccine offer and its timing, it seems useful to suggest a scheme to introduce the Meningococcal B vaccine. This is the recommended sequence of immunization (the days are suggestive and not mandatory): Hexavalent vaccine + Pneumococcal vaccine (PCV) at the start of the 3rd month of life (61st day of life), Meningococcal B vaccine after 15 days (76th day), Meningococcal B vaccine after 1 month (106th day), Hexavalent vaccine + Pneumococcal vaccine (PCV) after 15 days, at the start of the 5th month of life (121st day of life), Meningococcal B vaccine after 1 month, at the start of the 6th month of life vita (151st day of life), Hexavalent vaccine + Pneumococcal vaccine (PCV) at the start of the 12th month of age, Meningococcal B vaccine starting from the 13th month of life, Meningococcal C vaccine, always after the 1st year of life. **The third dose must be administrated at least 6 months after the second dose. ***The fourth dose, the last of the first cycle, must be administrated during the 5th - 6th year of age. It's also possible to use the adult formulation (dTap) at the start of the 5th year of life if parents receive adequate information high coverage rates are reached in adolescent populations. ****Subsequent booster every 10 years. *****In response to the outbreaks occurred in the past years, the catch-up of the susceptible individuals and an

active research of unvaccinated individuals (mop-up) is strictly recommended. ^Individuals without history of varicella: Administration of two doses of vaccine. The second administration 30 days after the first. ^^It's necessary to give two doses to the children who begin the administration during the second year of life. It's required just one dose if they begin the administration during the third year of life. One dose of PCV against a higher number of pneumococcal serotypes is recommended to unvaccinated children or children vaccinated with PCV7. Two doses are recommended for high-risk children. §One dose. Meningococcal C vaccine is administrated for birth cohort at the 13th–15th month of life. For the second birth cohort (12–14 years) one dose of Men ACWY vaccine is recommended for unvaccinated individuals and for the adolescent who were already vaccinated during childhood with the Men C or Conjugate Men ACWY vaccine. Meningococcal C vaccine could be administrated to at-risk individuals from the third month of life following a three dose scheme: the third dose after the 1st year of life. Administrate: two doses at 0 and 6 months (bivalent vaccine for individuals aged 9–14 years; quadrivalent vaccine for individuals aged 9–13 years), three doses at 0, 1 and 6 months (bivalent) or 0, 2 and 6 months (quadrivalent) for older individuals. Immunization with the seasonal vaccine for the individuals considered at risk by the Italian Minister of Health (Ministerial Circular). #Administration is recommended for one 65 years of age cohort. ##It's recommended to offer the vaccine universally, it's co-administrable with all the vaccines of the first months of life. Immunizations recommendations for high-risk groups: (1) dTap: number of doses depending if complete cycle or booster. For all pregnant women recommended during the third trimester of pregnancy (ideally at 28 weeks). (2) Hepatitis B: Pre-exposure: 3 doses (0, 1, 6 months). Postexposure: 4 doses (0, 2, 6 weeks + booster after 1 year). Immediate Pre-exposure: 4 doses (0, 1, 2, 12 months?). (3) Hib: for unvaccinated high risk subjects: number of doses to be decided on the basis of age and according to the product information template. (4) PCV: for children aged 0–5 years, PCV/PPSV after 5 year of age. (5) MMR: 2 doses with a minimum interval of 4 weeks; co-administration with monovalent varicella vaccine (V) or with quadrivalent vaccine (MMRV) is available/possible on the basis of age and of immunity against varicella. (6) Varicella: 2 doses with a minimum interval of 4 weeks; co-administration with monovalent varicella vaccine (V) or with quadrivalent vaccine (MMRV) is available/possible on the basis of age and of immunity against measles, mumps and rubella. (7) For high risk individuals it is recommended to offer Men ACYW and Men B, number of doses on the basis of age and according to the product information template. (8) HPV: recommended to subjects of all ages according to the product information template - number of doses on the basis of age and according to the product information template. (9) Influenza: for all ages according to the product information template - number of doses on the basis of age and according to the product information template. (10) Herpes Zoster: recommended to subjects aged 50 years. (11) Hepatitis A: number of doses on the basis of the product information template.

PREMATURITY AND IMMUNE SYSTEM

Premature neonates are defined by the World Health Organization, those born alive before the 37th week from the first day of the last menstrual period [174].

Moreover, prematurity is defined by the birth weight as follows:

Low birth weight infants (LBW) are those born weighing less than 2500 g;

Very Low Birth Weight *infants* (VLBW) *a*re those born weighing less than 1500 g;

Extremely Low Birth Weight infants (ELBW) *a*re those born weighing less than 1000 g.

The VLBW and the ELBW index accurately predicts the infant mortality rate. There is an important positive correlation between premature birth and IURG (intrauterine growth restriction) and low socioeconomic level.

Families with low socioeconomic status (SES) have relatively high incidences of maternal malnutrition, anemia and disease, inadequate prenatal care, drug addiction.

On the other hand, the premature birth of newborns whose birth weight is appropriate for the gestational age is linked to pathological conditions in the course of pregnancy such as a timely release of the placenta, or symptomatic bacterial infections in the amniotic fluid that can cause a preterm labor. This is due to bacterial antigens which are able to activate the production of local cytokines that induce premature uterine contractions and which subsequently lead to a premature birth.

Many studies have compared the functioning of the newborn's immune system with that of the adult.

In the fetus throughout pregnancy an active transport of IgG takes place through the placenta which guarantees to full term babies an immunoglobulin concentration comparable to that of the mothers, the other antibody classes do not cross the placental barrier even though the fetus can produce IgA and IgM in response to intrauterine infections.

In preterm infants, IgG levels in the umbilical cord are directly proportional to the gestational age. Studies concerning IgG antibodies directed against group B streptococcal antigens have shown that the ratio between the concentrations present at the level of the umbilical cord and the serum of the mother is equal to 1 in full-term baby and are reduced in newborns born at 32 and 28 weeks of gestation. Neonates, weighing less than 1500 g, frequently become hypogammaglobulinemics with a plasma IgG concentration of 200-300 mg/dl in the first week of life[175].

The complement system instead mediates bactericidal activity against certain pathogens and there is no transfer of complement factors from the mother.

In addition to significant hypogammaglobulinemia, the levels and functional capacity of components of the complement system are commonly decreased in newborns, particularly in preterm infants. The combined deficiency of immunoglobulins and complement factors results in reduced efficiency of opsonisation and contribute to infectious susceptibility [175].

Regarding the number of circulating neutrophils in full term and premature infants, it's been showed high, with a peak at the twelfth hour, returning to normal values by the 22nd hour.

The monocyte-macrophage system is globally normal both in term and in premature newborns, even if the function of macrophages of reticulo-endothelial system, especially the chemotactical function, is reduced.

According a study from 2015, monocyte are present in adequate numbers in preterm neonates, but are hypoactive after in vitro stimulation of TLRs.

As shown in Figure 13, production of IL-6 and TNF-α was substantially reduced in a subgroup of very preterm infants both in monocytes, compared to term neonates or adults.

Dendritic cells are the main source of IL-12/23p40 in human blood. IL-12 and IL-23 are two cytokines having important roles in the differentiation of T helper lymphocyte immune responses. Similar to the reduced IL-6 and TNF-α responses observed in preterm monocytes, production of IL-12/23p40 was also markedly reduced in dendritic cells from both term and preterm neonates (figure 14)[177].

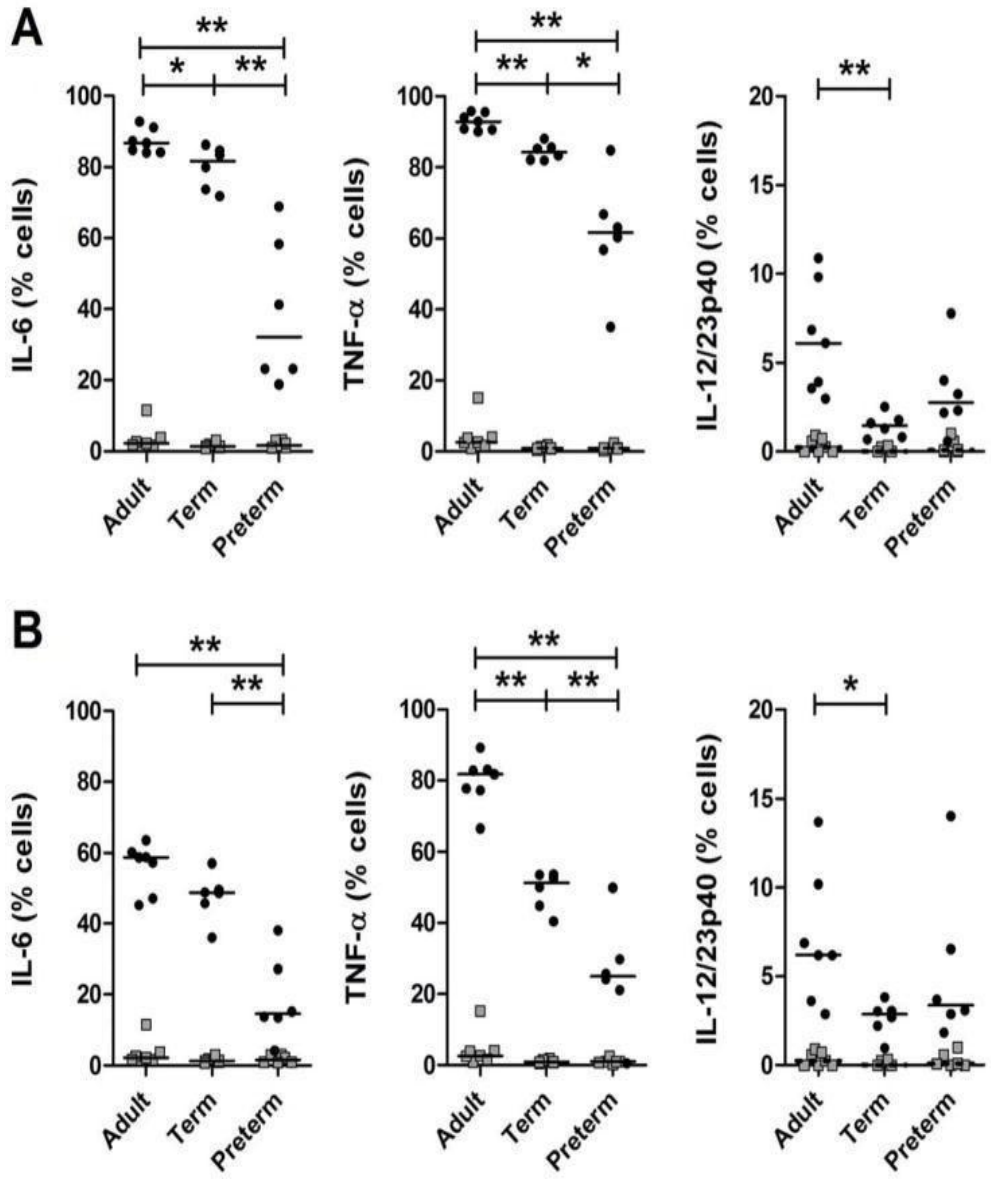


Figure 14. Single-cell cytokine production in innate immune cells Single-cell (A) LPS- and (B) R848- induced cytokine responses (black circles) in monocytes (for IL-6 and TNF-α) and in dendritic cells (for IL-12/23p40) in very preterm neonates (mean gestational age and birth weight: 27.2 weeks and 1063 g, respectively; median postnatal age: 13 days, range 7 to 23), term neonates and adults, compared to unstimulated responses (gray squares); *p<0.05; **p<0.01.

According to this study there are postnatal responses to stimuli such as LPS and R848, deeply attenuated in premature babies, consistent with responses measured in cord blood. Low expression of TLR is present on surface of the mononuclear cells of premature infants, with TLR2 and TLR4 increasing in the first 2 months of age.

TLRs play a critical role in protecting against microbes in early life, as indicated by an increased vulnerability to specific bacterial and viral infections in humans with loss of function mutations along these molecular pathways.[178] The risk of infections in these patients is greatest during the neonatal/early childhood period, underlining the importance of these receptors for immune protection during infancy [176].

As shown by previous epidemiological studies, the incidence of sepsis increases significantly in these newborns shortly after birth (during the first week) and then decreases over weeks.

Other reasons behind the growing vulnerability of these newborns to sepsis are high invasiveness and microbial colonization of skin and mucous membranes after birth.

Therefore the ability of very preterm neonates to respond to PAMP (Pathogen Associated Molecular Patterns) is profoundly attenuated over the first month after birth.

Prematurity, neonatal sepsis and histological corioamnionitis have been associated with low monocyte major histocompatibility complex (MHC) class II expression [179].

Serial monocyte MHC class II expression have revealed a fall by day 2 in all preterm neonates, with the degree being influenced by both prematurity and sepsis, and incomplete recovery by day 7.

Natural killer (NK) cells are a subgroup of lymphocytes with cytolytic action against virus-infected cells and are covered over the entire surface with antibodies by a process called antibody- dependent cell-mediated cytotoxicity (ADCC).

The percentage of NK cells is lower in term newborns as compared to adult (CD3-CDI6+CD56+; 4.8 versus 15.5%) [180].

T lymphocytes, in particolar helper T cells subset (CD4 positive), play a fundamental role in the regulation of the immune system.

They are indicators of global immune function since they are involved in the modulation of adaptive immunity, both humoral and cell-mediated, and also play an important role in controlling the innate immune response.

From the 19th week of gestation, T lymphocyte subpopulations increase progressively and continue to grow after birth up to a peak at the age of 6-9 months of life.

They decrease afterwards, reaching normal adult levels at about 6-7 years of age.

In newborns, CD4 + T cells represent the highest percentage of T cells compared to adults. In fact, CD8 + T lymphocytes are less both in terms of absolute number and percentage of total T cells.

In premature infants, levels of absolute numbers of total helper T cells subset (CD4+) at birth are higher than those of term neonates and inversely correlated with gestational age. The CD8- positive cells are lower in neonates but their levels did not correlate with gestational age [181].

In the study by Feza M. et al. age-related changes in lymphocyte subpopulations of human blood have been examined but do not reflect cellular function [182].

To assess the adaptive response of the immune system in preterm infants CD4 + T lymphocyte response to in vitro stimulation by a non-specific mitogen PHA has been

analyzed.

The production of ATP, an important final lymphocyte metabolite and an optimal indicator for the evaluation of lymphocyte cell function was established.

It was found that premature infants have a reduced ability to activate CD4 + T cells compared to adults. As matter of fact the ATP values at birth are low and, despite a propensity towards higher values over time, they remain significantly lower up to 30 days of life.

Moreover, it is known that all newborns, especially premature ones, have deficiencies in innate and adaptive immunity. In fact, many studies have shown lower concentration of cytokines such as TNF- α, IFN- α, IL-4, IL-5, IL-10, IL-15 and IFN- γ in blood from premature infants compared to adults. However, the production of cytokines is an indirect indicator of cellular function. This study establishes the dysfunction of CD4 + T cells in the early stages of T cell activation.

Naıve T cells are characterized by the expression of CD45RA and CCR7, the costimulatory receptors CD28 and CD27, and the lack of expression of cytolytic molecules. Long-lived central memory T cells home preferentially to lymph nodes because they express the lymph node-homing markers CD62L and CCR7 and share several phenotypic properties with naive T cells but do not express CD45RA. Central memory T cells, as opposed to naive T cells, can rapidly differentiate into cells endowed with effector function upon exposure to antigen [43], can upregulate CD40L to a greater extent than naıve cells and are characterized by their ability to proliferate and secrete high levels of IL-2 [183].

In a study by J C Walker et al study the differences in the various components of the immune system in groups of full-term, late preterm and very preterm newborns, were observed. According to this work, no significant differences were observed regarding the naïve T cells among the different groups, although very preterm infants showed a slightly lower number than the other infants.

The Authors suggest that this difference may be due to a reduction in hemoglobin production or a decrease in the peripheral expansion of T lymphocytes due to the obvious limitations in their functionality in very preterm newborns [184].

In full-term infants the production by thymus of naïve T lymphocytes has been shown to be normal.

Most B lymphocytes consist mainly of naive cells in both full-term and preterm infants and reduced numbers of these lymphocyte cells were observed in infants compared to adults.

More recently, memory B cells have been shown to express Toll-like receptor 2 (TLR2) [185].

Extremely low frequencies of B lymphocytes expressing TLR-2 and TLR-4 have been observed in all newborns.

Early birth is generally caused by negative perinatal factors such as maternal pre-eclampsia or intrauterine infections. These factors can influence the concentration of lymphocyte subpopulations in neonatal umbilical cord blood.

A growing number of studies indicate that the incidence of various medical problems, both short and long term ones, is greater in late preterm infants than in full term infants [186-187].

During the birth hospitalization, infants bornat 34 0/7 to 36 6/7 weeks' gestation compared with term infants experience more difficulties with feeding (32% versus 7%), hypoglycemia (16% versus 5%), jaundice (54% versus 38%), temperature instability (10% versus 0%),apnea (6% versus <0.1%), and respiratory distress (29% versus 4%).Late preterm infants also receive intravenous fluids (27% versus 5%), evaluations for sepsis (37% versus 13%), and mechanical ventilation (3.4% versus 0.9%) more often than their term counterparts. Late preterm infants are 3.5 times more likely to have two or more of these problems than term infants. Because of these medical illnesses and management requirements, many of these infants need specialty care and hospitalizations beyond 5 nights in neonatal intensive care units. Admission to intensive care unit is inversely proportional to gestational age. In a large health care system, 88% of infants born at 34 weeks' gestation, 12% born at 37 weeks'gestation, and 2.6% born at 38 to 40 weeks' gestation were admitted to an intensive care unit. Duration of hospital stay is also inversely propor-tional to gestational age [188].

The increased risk of bacterial infection in preterm infants is influenced by a series of triggering factors such as the prolonged request for admission to an intensive care unit, the greater use of mechanical ventilation, parenteral feeding and intravenous accesses that violate the physical barriers of organism and facilitate invasion by nosocomial pathogens.

As mentioned before, newborns, particularly preterm infants, often have the appropriate tools to deal with extracellular pathogens, as shown by the numbers relative to most of the cells of the innate immune system and the expression of recognition molecole. However, the appropriate number of cells does not indicate their correct functionality.

For this reason prenatal vaccination is recommended to pregnant mothers and several studies highlight their effectiveness[189].

Placentally transmitted anti pertussis immunoglobulins play an important role in first line defence against invading pathogens.

Preterm neonates benefit from second- rather than third-trimester Tdap maternal immunization [190]. These results are unexpected, as the greater efficacy of placental transfer during the third trimester is well established. This transfer is mediated through the neonatal Fc receptor (FcRn), expressed in the syncytiotrophoblast as of gestional week (GW) 13, when antibody transfer begins.

FcRn-mediated transport efficacy, which is both active and saturable, slowly increases with time as the cytotrophoblast becomes discontinuous. Consequently, the proportion of fetal to maternal total immunoglobulin G (IgG) antibody concentrations at birth increases steadily from 10% (GW 17–22) to 50% (GW 28–32) to ≥100% of maternal concentrations at term. Following early pregnancy vaccination, maternal antibodies are thus likely transferred to the fetus with lower efficiency. However, this reduced daily transfer is compensated by the longer total transfer time, resulting in antibody accumulation in the fetal circulation. This longer transfer time may be the key element of the higher antibody levels following earlier maternal immunization.

Nevertheless, the half-life of anti-PTX antibodies is short. This suggests an additional mechanism protecting IgG antibodies from degradation during the long period of transfer to placental level. The catabolism vs recycling of serum IgG occurs in endothelial cells: only FcRn-bound IgG antibodies are protected from lysosomal degradation and transported back into the serum. As FcRn-mediated transport is saturable, the catabolism of IgG is faster at higher IgG serum concentrations. We hypothesize that the low concentration of fetal total IgG does not saturate the FcRn receptors, such that most maternal IgG antibodies successfully bind to FcRn and are largely protected from degradation.

AIMS OF THIS STUDY

Pertussis is a highly contagious infectious disease caused by the bacterium *Bordetella pertussis.* It affects the upper airways with symptoms which, at the beginning, are indistinguishable from those of normal cold. Subsequently, the symptoms are characterized by the typical tussive accesses, by the post-inspiration scream with closed glottis, which can cause apnea phases. The disease affects all ages, but mainly affects children under the age of 5 years [1,2].

To date, this disease is preventable by vaccination. In Italy 3 doses of hexavalent vaccine are offered at the 3, 5 and 13 months of age [13].

At pre-school age, a booster dose is administered, followed by booster doses every 10 years.

This vaccination schedule is offered to all babies born at term, while for premature babies the vaccination schedule is not uniform in all Italian regions [173].

In fact, some regions give 4 doses, instead of 3, to preterm infants because it is assumed that the immune system of preterm infants is less developed and therefore at greater risk of contracting the disease.

In Italy, the introduction of acellular vaccine against whooping cough in 1995 has made it possible to significantly reduce cases of disease and deaths due to the disease itself: the data for 2010- 2013 indicate a drop in the burden of disease of 97.6%.

Unfortunately, the number of cases of whooping cough is currently increasing in children, adolescents and adults [193]. There are basically three reasons for this phenomenon.

First of all, in Italy, as in the rest of Europe, there is a drop in vaccination coverage. Vaccination coverage for whooping cough is below 95% in all age groups monitored (24, 36 months and 6, 16 and 18 years)
[192].

It is also known that the immunity resulting from both vaccination and naturally contracted disease is not long-lasting: as a matter of fact, after 5 years from the last dose of the vaccine, the risk of getting sick with whooping cough in properly vaccinated subjects increases by 42% each year [191].

A third explanation for the increase in pertussis cases is the selective pressure induced by vaccination. The use of pertussis vaccines may have selected mutated bacteria that express antigens not included in the available vaccines. The pertussis vaccines currently on the market contain one or more antigenic determinants crucial to the induction of the immune response: pertussis toxin (PTX), filamentous hemagglutinin (FHA), pertactin (PRN) and fimbrias (FIM). These differ not only in the number of components 2 (PTX and FHA), 3 (PTX, FHA and PRN) or 5 components (PTX, FHA, PRN and FIM 2 and 3), but also in the amount of antigens contained [169, 170, 171].

The mutation of one or more of these antigens, caused by immunological pressure, could play a decisive role in the increase in cases of whooping cough in general and also in subjects already properly vaccinated [27].

It is therefore clear that continuous monitoring over time is crucial to understand which pertussis strains are in circulation.

In Tuscany, since 2010, the diagnosis of infection caused by whooping cough has been carried out at the Immunology Laboratory of Meyer Children's Hospital. The immunology laboratory is the regional reference centre for invasive bacterial diseases and manages the regional register of all cases of pertussis-related disease.

My study aims, on the basis of data collected from 2010 to 2019 by the regional reference center of invasive bacterial diseases, to analyze the epidemiology of pertussis infection in the paediatric population of Tuscany, with the aim of defining some of the main risk factors for both the occurrence of the infection and for the occurrence of serious infections that require hospitalization.

In detail, we will analyse the epidemiological characteristics of the subjects presenting the infection in order to deduce the best vaccination strategy. Through the knowledge of the incidence of whooping cough in preterm births we could obtain useful indications to be able to standardize the national vaccination calendar, even for this particular group of children.

Finally, my study aims at studying the strains of *B. pertussis* that circulate in Tuscany. In fact, we want to understand if in Tuscany, as in most of Europe, new varieties of *B.*

pertussis are emerging.

To achieve this goal, allele variants of genes coding for virulence factors used in the acellular vaccine were studied in 71 pharyngeal swabs from infected patients collected in Tuscany from 2010 to 2019.

MATERIALS AND METHODS

This study includes patients aged between 0 and 18 years with *Bordetella pertussis* infection, which was diagnosed between 2010 and 2019 at the Paediatric Immunology Laboratory of the Meyer Children's Hospital.

The cases were selected retrospectively thanks to the National Register of Invasive Bacterial Diseases of Pediatric Immunology Laboratory of the Meyer Children's Hospital.

The positivity of the infection was defined by the presence of typical clinical symptoms and by the positivity of Real time-PCR at *Bordetella pertussis* (194 swab samples).

Clinical information on prematurity, severity of infection, length of hospitalization stay, oxygen requirements was obtained by reviewing hospital records, discharge letters, telephone interviews with family paediatricians and parents.

Finally, in the present study, the DNA of 70 of these clinical samples were subjected to molecular characterization.

PCR AMPLIFICATION

Whooping cough laboratory confirmation was obtained by Realtime-PCR (RT-PCR). Respiratory samples were obtained using nylon nasopharyngeal swabs (ESwab, COPAN, Murrieta, California). Bacterial DNA was extracted for molecular test using the QIAamp DNA Mini Kit (Qiagen,Hilden,Germany) according to the manufacturer's instructions. Detection of *B pertussis* was performed using RT-PCR, amplifying the ptx genomic targets (pertoxfor,5'- cggatgaacacccataagcat- 3'; pertoxrev, 5'- cgatcaattgctggaccattt- 3'; pertoxprobe 5'- cccgattgaccttcctacgtcgactc- 3'). All reactions were performed in duplicate. The reaction mix contains 6,25µl 2X TaqMan Fast Universal PCR Master Mix No AmpErase UNG (Applied Biosystems, Foster City, California), the primers at a concentration of 400 nM, the probe, marked in FAM, at a concentration of 200 nM and 3µl of extracted DNA. RT-PCR was performed on an Applied Biosystems 7500 Fast RT-PCR System platform. The DNA was amplified using the following cycle parameters: 95°C for 2 min followed from 45 cycles of a two-stage temperature profile 95°C for 3 s and 60°C for 30 s. A sample was considered negative if there was no increase in fluorescent signal before RT-PCR cycle 45.

SEQUENCE-BASED TYPING

Molecular characterization of *B. pertussis* was performed by sequencing the ptxA, ptxP, and prn with a PCR amplification of chromosomal DNA followed by direct sequencing of the PCR products. Conditions for amplification of genes were as follows. The prn gene was amplified in 25,5 µL containing 2,5 µL MgCl2 10X, 5 µL GC rich solution 5X, 2 µL dNtps 2,5 mM, 0.5 µmol of each primer and 0,2 µL of Fast Start Taq Dna polymerase 5U/ µL and 2,5µl of extracted DNA. The reaction mixtures were preheated at 95°C for 3 min, and 36 amplification cycles were performed in a Mastercycler nexus gradient eco, using the following program: 20 s at 95°C, 30 s at 55°C, and 1 min at 72°C. The last cycle was concluded with reaction for 7 min at 72°C.

The ptxA gene was amplified in 25 µL containing 2,5 µL Q-solution 5X, 12,5 µL Multiplex PCR Master Mix 2X, 0,5 µmol of each primer and 5µl of extracted DNA. The reaction mixtures were preheated at 95°C for 3 min, and 30 amplification cycles were performed in a Mastercycler nexus gradient eco, using the following program: 15 s at 95°C, 15 s at 59°C, and 1 min at 72°C. The last cycle was concluded with reaction for 10 min at 72°C.

The ptxP gene was amplified in 25 µL containing 2,5 µL Q-solution 5X, 12,5 µL Multiplex PCR Master Mix 2X, 0.5 µmol of each primer and 5µl of extracted DNA. The reaction mixtures were preheated at 95°C for 15 min, and 40 amplification cycles were performed in a Mastercycler nexus gradient eco, using the following program: 5 cycles of 15 s at 95°C, 30 s at 70/68/66/64/62°C (decreasing each cycle) and 1 min at 72°C; followed by 35 cycles of 15 s at 95 °C, 30 s at 60 °C and 1 min at 72°C. After the last cycle, a final step of 10 min at 72°C was added.

The primers sequences used to amplify and sequence the prn, ptxA genes and ptxP are indicated in table 4. In order to sequence the different regions where the pertactin gene can have allele variants different primers were used, while for the pertussis toxin promoter sequencing primers annealing with sequences in the upstream and downstream genes were used.

	Primers
prn Pfw	TGTCTCTGTCACGCATTGTC
prn Afw	GCCAATGTCACGGTCCAA
prn Arv	GCAAGGTGATCGACAGGG
prn Brv	CGGATTCAGGCGCAACTC
prn Bfw	AGCTGGGCGGTTCAAGGT
prn Prv	ATGCCGTTGGTGTGTACCGT
ptxA S1fw	TAGGCACCATCAAAACGCAG
ptxA S1rv	TCAATTACCGGAGTTGGGCG
ptxP fw	AATCGTCCTGCTCAACCGCC
ptxP rv	GGTATACGGTGGCGGGAGGA

Table 4. Primers were derived from published sequences of prn, ptxA and ptxP [222,223].

The result of the amplification was checked through electrophoresis in agarose gel 2,5% where a Fluorescent Nucleic Acid Stain 20.000X was added. The electrophoresis was run in a TAE buffer at 95 Volt.

This DNA was then sequenced on both strands with the primers used for amplification. Sequence reactions were carried out with a BigDye™ Terminator v3.1 Cycle Sequencing Kit and the products were analysed on a model ABI 3130xl DNA sequencer.

STATISTICAL ANALYSIS

All statistical analyses were performed using the GraphPad and MedCalc Software.The Fisher's test was used to statistically compare and the Chi-squared test was used to compare proportions. Differences between means or proportions were considered to be statistically significant when the p-value was below 0.05. In addiction, all results were expressed as Odds ratios (OR) and 95% confidence intervals (CI).

RESULTS

In the swab sample of 194 patients who had whooping cough obtained from the analysis of the Register of Invasive Bacterial Diseases, 3 cases came from external analysis laboratories, while 191 cases came from hospital wards: 125 cases from Meyer Children's Hospital, while the remaining 69 from other hospitals in Tuscany. The SDO data (Hospital Discharge Cards) for the period 2010 - 2019 of diagnosis of discharge of pertussis in Tuscany reported 194 cases, confirming that all cases of pertussis occured in Tuscany in the study period have been diagnosed by Pediatric Immunology Laboratory of Meyer Children's Hospital.

DISEASE INCIDENCE AND HOSPITALIZATION RATE

I divided the cases by year of infection: 3 (3/194 1.54%) cases in 2010, 4 (4/194 2.06%) cases in 2011, 7 (7/194 3.61%) cases in 2012, 17 (17/194 8.76%) cases in 2013, 23(23/194 11.86%) patients in 2014, 26 cases (26/194 13.40%) in 2015, 32 patients (32/194 16.49%) affected in 2016, 59 patients (59/194 30.41%) in 2017, 3 cases (3/194 1.54%) in 2018 and 20 cases (20/194 10.30%) in 2019.

The number of residents in Tuscany in the age group 0-18 years divided by year can be found in the Excel Tables on the website of the Region of Tuscany. Thanks to this information, I calculated the incidence by dividing the number of cases on population living in Tuscany from 0 to 18 years and multiplying by 100000 ab. The pertussis incidence in 2010 was 0.51 x 100000 ab, in 2011 0.68 x 100000 ab, in 2012 1.17 x 100000 ab, in 2013 2.81 x 100000 ab, in 2014 3.80 x 100000 ab, in 2015 4.31 x 100000 ab, the incidence in 2016 5.32 x 100000 ab, in 2017 9.81 x 100000 ab, in 2018 0.50 x 100000 ab and in 2019 3.19 x 100000 ab.

In the sample of 194 cases of whooping cough I found 48 (48/194 24.74%) hospitalized patients and divided them according to the year of infection. I calculated the rate of hospitalization for pertussis in the population living in Tuscany in the age group 0-18 years, from October 2010 to October 2017, then multiplying it by 100000 ab. In 2012 the hospitalization rate for whooping cough was 0.34 x 100000 ab, in 2013 0.66 x 100000 ab, in 2014 2.48 x 100000 ab, in 2015 2.15 x 100000 ab, in 2016 0.83 x 100000 ab, in 2017 1.83 x 100000 ab, in 2019 0.16 x 100000 ab (table 5).

Year	cases	resident population in Tuscany (0-18 years)	incidence of pertussis cases Meyer Hospital x 100000 ab.	hospitalizations	hospitalization rate x 100000 ab.
2010	3	592257	0,51		
2011	4	588526	0,68		
2012	7	595797	1,17	2	0,34
2013	17	604851	2,81	4	0,66
2014	23	605536	3,80	15	2,48
2015	26	603647	4,31	13	2,15
2016	32	601160	5,32	5	0,83
2017	53	601160	9,81	11	1,83
2018	3	597780	0,50		
2019	20	626092	3,19	1	0,16

Table 5. Cases of whooping cough in Tuscany and distribution according to year, incidence and hospitalization.

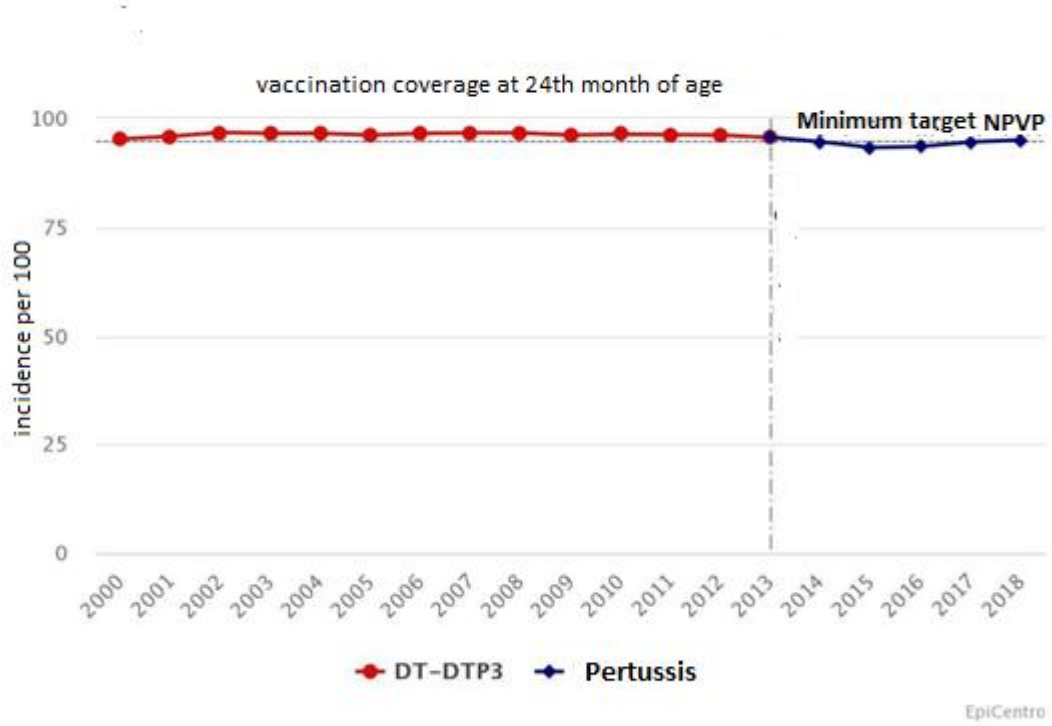


Figure 15 [226] Vaccination coverage against whooping cough.

The number of cases of whooping cough, which I obtained through the Register of Invasive Bacterial Diseases of the Immunology Laboratory of Meyer Children's Hospital, increased from 2010 to 2016 by about 10 times in parallel with a decrease in vaccination coverage during the same period. In fact in 2010 the vaccination coverage for whooping cough was 96.4% and it was reduced to 94.6% in 2014 and until 93.5% in 2016. The latest updates provided by Italian Institute of Health, showed an increasing trend in vaccination coverage from 2017 to 2018. In 2018, vaccination coverage was estimated to be 95.07%. At the same time, the number of patients with whooping cough has fallen dramatically (figure 15).

SAMPLE CHARACTERISATION BY AGE

In the sample of 194 patients who had whooping cough in the Tuscany Region (101 female patients 52.06% and 93 male patients 47.94%) 119 cases were found to be less than one year old (119/194 61.34%), 75 cases of children over the first year of life (75/194 38.66%) in particular 38 cases had an age of 1-5 years (38/194 19.59%), 16 cases in the age group 5-10 years (16/194 8.25%) and 21 cases of whooping cough between 10-18 years (21/194 10.82%) (figure 16).

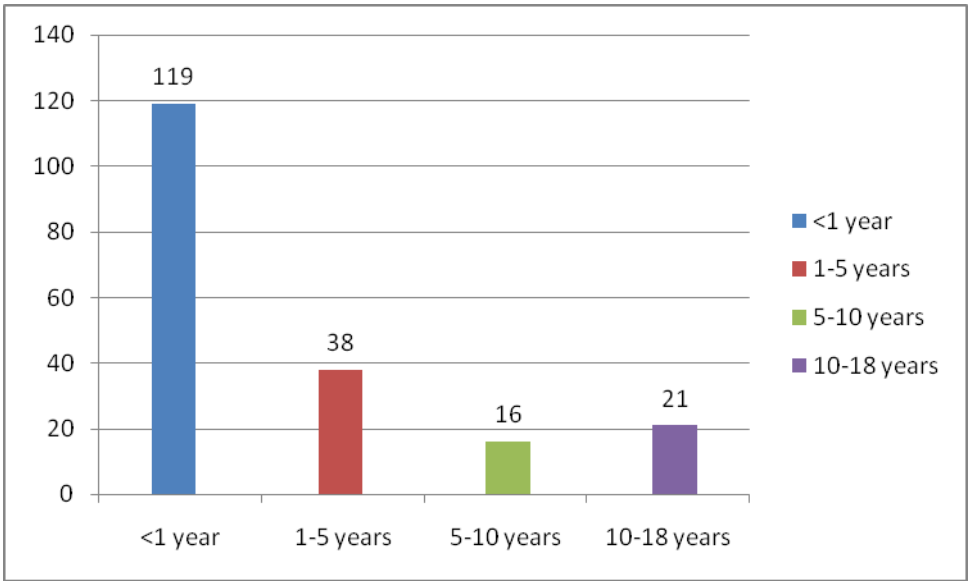


Figure 16. Distribution of pertussis cases by age group.

More in detail, examining the 119 cases of whooping cough under the year of life we found 36 cases (36/119 30.25%) between 0-1 month, 36 cases (36/119 30.25%) between 1-2 months, 17 cases (17/119 14.29 %) between 2-3 months, 15 patients (15/119 12.61%) between 4-5 months, 8 cases (8/119 6.72%) between 6-7 months, 4 patients (5/119 4.20%) between 8-9 months and 3 cases (3/119 2.52%) between 10-12 months (figure 17).

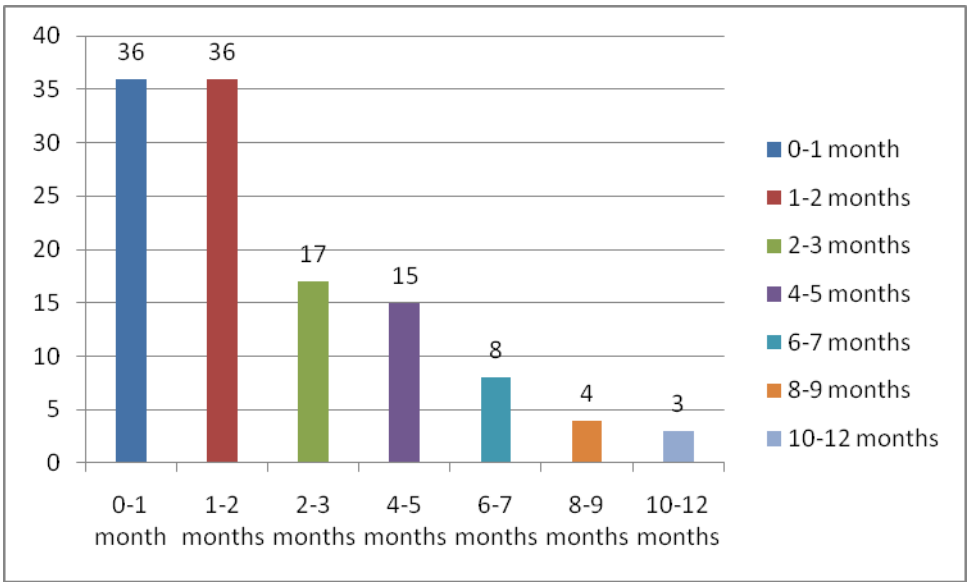


Figure 17. Distribution of pertussis cases by age group, under 12 months of age.

Among the subjects included in this study (194) we managed to obtain all the information sought in a sample of 99 children, so for all future results we will refer to this group of children that we were able to study in a comprehensive way, in this subgroup of patients, 57 cases (57/99 57.58%) presented the infection in the first year of life, 24 patients (22/99 22.22%) between 1-5 years, 8 cases (8/99 8.08%) between 5-10 years and 10 patients with whooping cough (10/99 10.10%) between 10 and 18 years (figure 18).

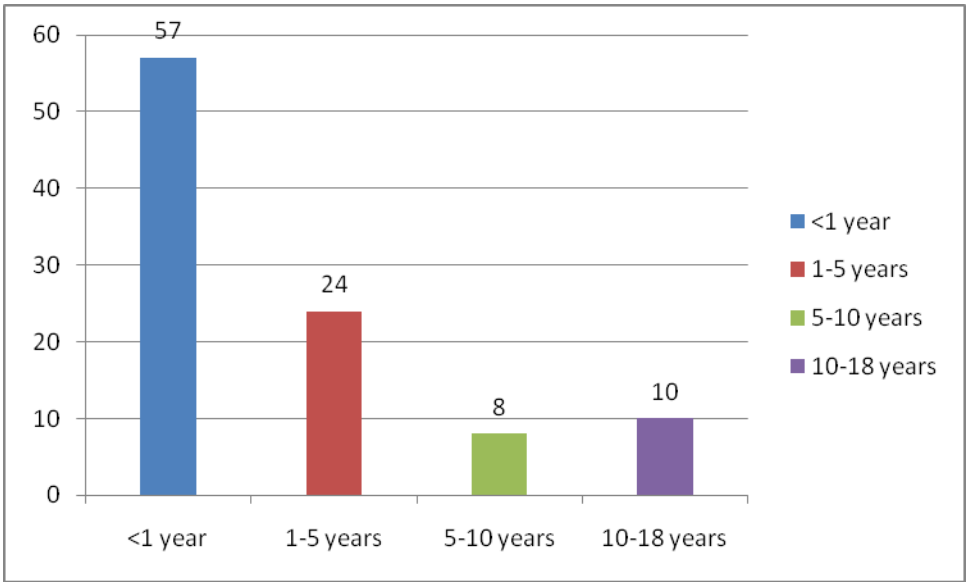


Figure 18. Distribution of pertussis cases by age group, in the subgroup of 91 patients.

More in detail, analyzing the 57 patients under one year of life, 18 cases (18/57 31.58%) were in the age group between 0-1 month of life, 12 cases of whooping cough (12/57 21.05%) between 1-2 months of life, 7 patients (7/57 12,28%) between 2-3 months of life, 11 cases (11/57 19,29%) between 4-5 months of life, 5 patients (5/57 8.77%) between 6-7 months of life, 2 cases (3/57 5.26%) between 8-9 months of life, 2 cases (2/57 3.51%) between 10-12 months of life (figure 19).

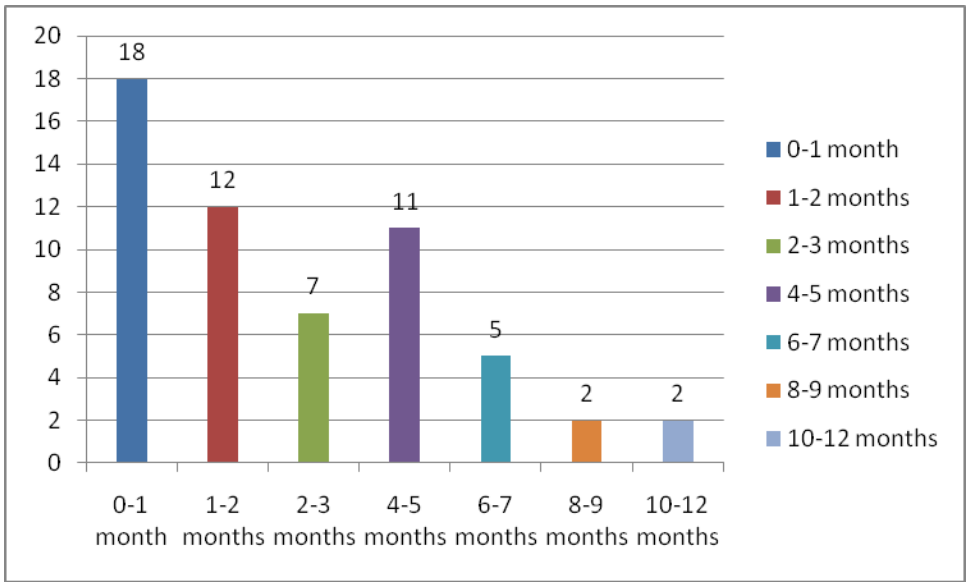


Figure 19. Distribution of pertussis cases by age group, under 12 months of age in the subgroup of 99 patients.

SAMPLE CHARACTERISATION BY GESTATIONAL AGE AND VACCINATION STATUS

	PRETERM BIRTHS	FULL TERM BIRTHS	TOTAL PATIENTS
GENERAL POPULATION OF TUSCANY	2293	23093	25386
SELECTED SUBGROUP IN THE STUDY POPULATION	9	90	99
TOTAL PATIENTS	2302	23183	25485

Table 6. Births in Tuscany general population and the number of premature births.

In table 6 I have inserted the data of births in Tuscany general population, 25386, and the number of premature births 2293 (9% of births).
90 subjects were term-born (90/99 90.91%), the remaining 9 patients (9/99 9.09%) were preterm- born children, so the percentage of premature patients with whooping cough in the sample is 9.09%. The difference between the incidence of prematurity in our sample is not statistically significant compared with the incidence in the general population (Chi-squared test 0.00 with p:0.98, degree of freedom 1).

In our sample of children (99), we have 9 (9/99 9.09%) premature births and 90 (90/99 90.91%) term births. In particular, 6 of the cases born preterm were older than one year (6/57 10.53%), the other 3 cases born preterm were less than one-year-old (3/42 7.14%).
As for the anti-pertussis vaccination status of the 99 children in the sample: 21 children (21/99 21.21%) had received full vaccination according to the vaccination calendar for their age.

24 patients were incompletely vaccinated, however they had received at least one dose of the vaccine (24/99 24.24%) while 54 children were not vaccinated at all (18 were not

vaccinated because they were less than 60 days old at the time of infection). In this sample of 78 cases not adequately vaccinated, 3 were premature (3/78 3.85%).

40 patients had received at least one dose of vaccine (21 patients had received complete schedule and 24 patients had received at least 1 dose). Among these, we analysed how many had kept to the vaccination schedule and how many had not.

To establish the criterion of appropriateness of the vaccine doses in relation to the age of the child at the time of infection, we have established a maximum of 4 months for the 1st vaccine dose, 6 months for the 2nd vaccine dose and 13 months for the 3rd vaccine dose.

Of the 21 children who had received the complete primary cycle, 13 were on time, and 8 had not received their 3rd dose within 13 months of birth. For the 24 children who had not received the full primary cycle, 16 children (16/24 66.67%) had received the doses within the overwritten time. 8 children (8/24 33.33%) received only one or two doses and were late compared to expected vaccination schedule.

In the subgroup of 21 patients vaccinated with 3 doses we analyzed the period in which they were affected by whooping cough. We used the date of pharyngeal swab collection for the search of *Bordetella pertussis* positive result and the date of the last vaccination and we observed that between the two dates there was an average time of about 4 years and a half, so we considered how this period is the average time of loss of vaccination effectiveness after which the vaccinated subjects may be susceptible to infection again (figure 20).

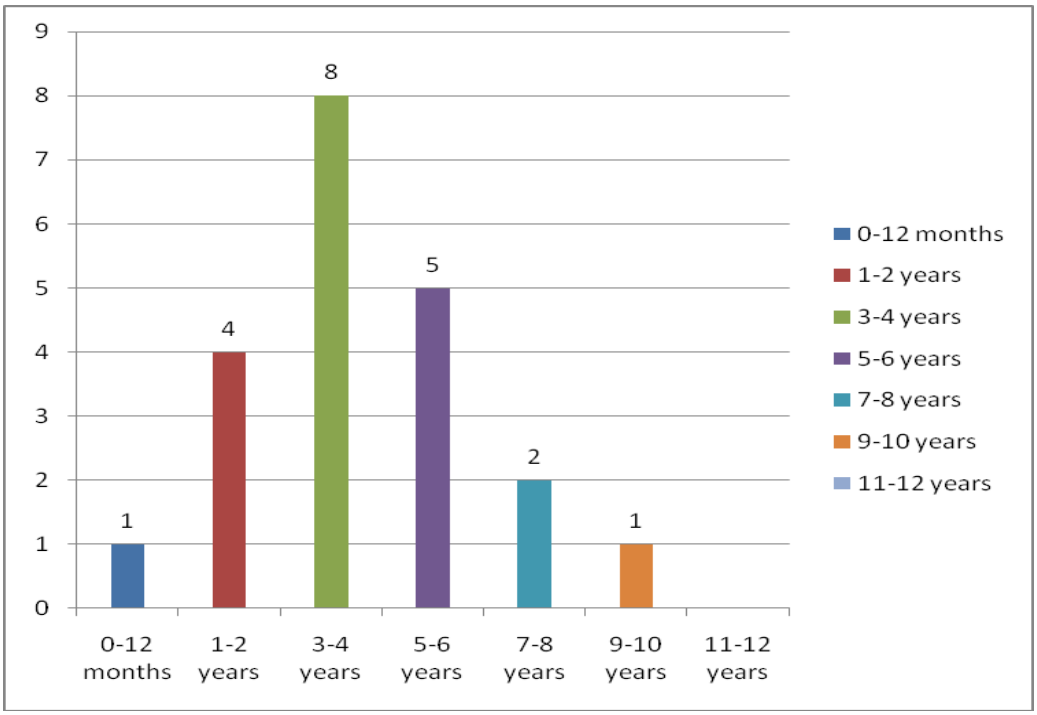


Figure 20. Distribution of cases according to latency period between date of last vaccination dose and date of infection, in a subgroup of 21 fully vaccinated patients.

With regard to the 9 preterm children and their vaccination status, 2 children had been vaccinated adequately according to age, one child had received the last dose 12 years before the infection, 1 child had not received 2nd dose, 1 child had received 3rd dose inadequately, while 4 children had not been vaccinated.

QUALITATIVE CHARACTERISATION OF THE SAMPLE: HOME THERAPY, HOSPITALIZATION, INTENSIVE CARE.

The number of patients hospitalized is 48 (48/99 48.48%) of which 6 are premature (6/48 12.50%). The patients who only needed home therapy were 51 (51/99 51.51.83%), of which 3 were preterm (3/51 5.88 %) and 48 (48/51 94.11%) were born on term. Being born preterm does not represent a risk factor for whooping cough requiring hospitalization (Fisher's test P=0.309; OR=2.29; 95%CI from 0.54 to 9.71) (figure 21).

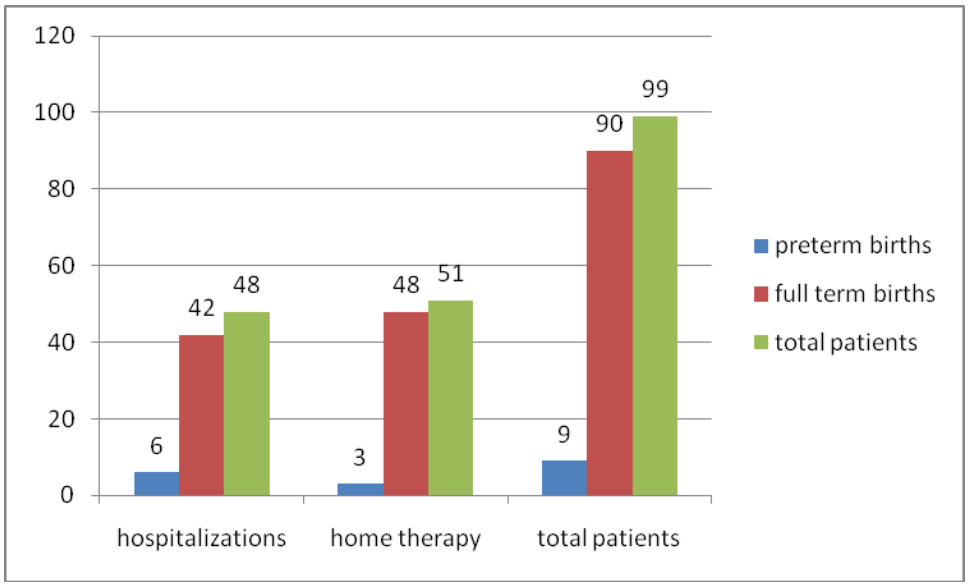


Figure 21. Distribution of cases of whooping cough according to need of hospitalization, divided into preterm and term births.

Considering the age of onset of disease, of the 48 hospitalized 42 (42/48 87.50%) had less than one year of life, while 6 (6/48 12.50%) had more than one year of life. In the group of non- hospitalized patients, 15 (15/51 29.41%) had less than one year of life, while 36 (36/51 70.59%) had more than one year of life. Age is a risk factor for severe pertussis infection In accordance with literature, in our study the children less than 1 year required hospitalization (Fisher's test P<0.0001; OR= 16.8; 95%CI from 5.9 to 47.82) (figure 22) more frequently than the rest of the cohort.

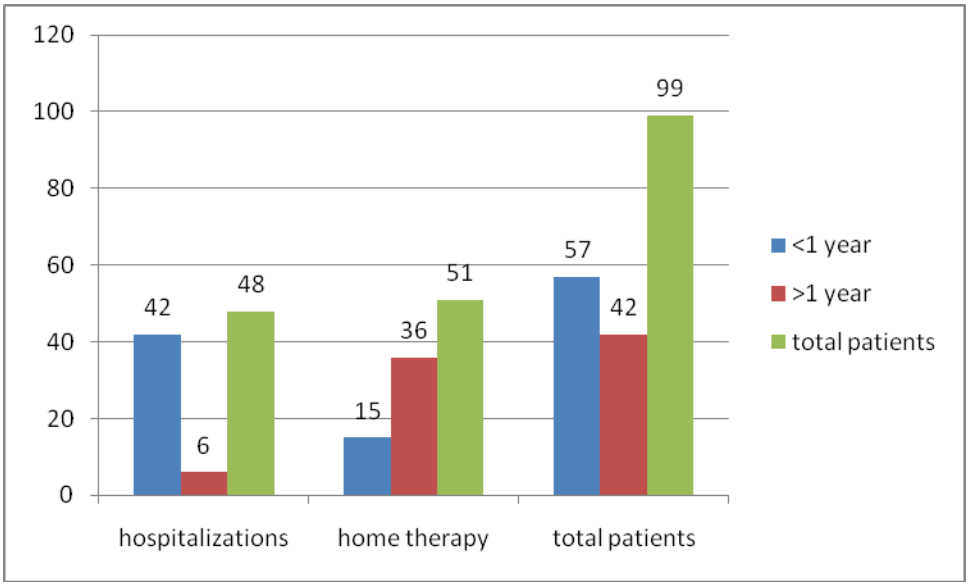


Figure 22. Distribution of pertussis cases according to the need of hospitalization, divided according to the age of onset of the infection.

Also 6 patients aged more than 1 year needed hospitalization, and among these patients 4 had another comorbidity (1 case was admitted after chemotherapy for acute lymphoblastic leukemia, 1 case was admitted for Kawasaki's disease, 1 case was admitted for rheumatic disease, 1 case had performed chemotherapy for medulloblastoma and another case was a late preterm who had not received a vaccine in more than 10 years).

Of all patients with whooping cough who needed to be hospitalized, 17 patients received at least one dose of the vaccine, in 14 cases respecting the indications for the age calendar.

16 patients had not received any dose even though they were all older than 60 days. 2 patients had received the complete cycle, but 1 case had received the last dose more than 10 years before the infection and 1 case had performed chemotherapy cycles for acute lymphoblastic leukemia before the infection. The difference between vaccinated and unvaccinated patients in the group of hospitalized and non-hospitalized patients was significant (Chi-squared test 3.042 with 1 degress of freedom, P=0.0406; OR=2.21; 95%CI from 0.98 to 4.98). (figure 23)

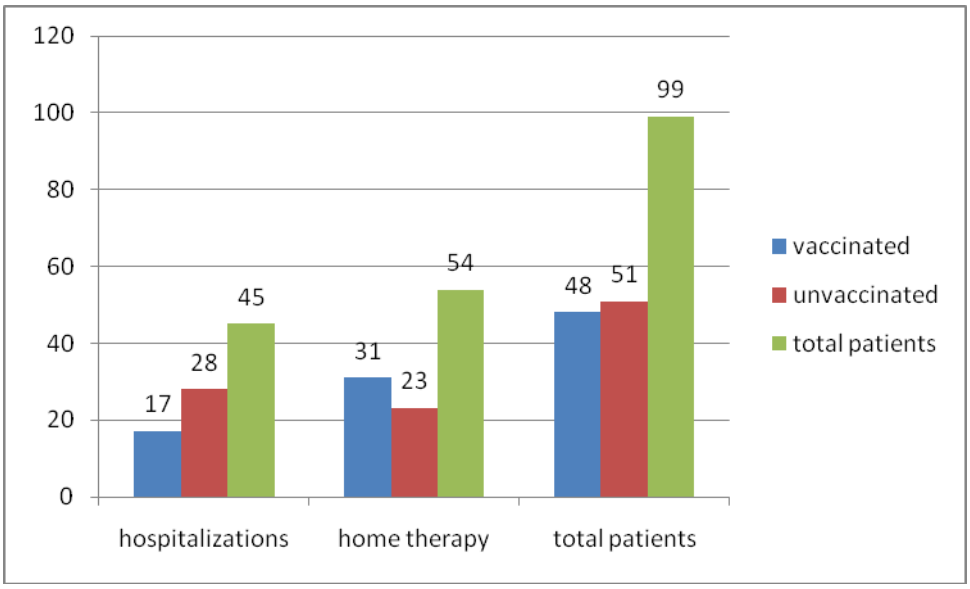


Figure 23. Distribution of pertussis cases according to to the method of treatment required, divided into vaccinated and non-vaccinated patients.

Regarding analyze the sample of hospitalized patients (48/99 48.48%), 15 had requested therapy with O_2 (15/48 31.25%), 16 (16/48 33.33%) had no need for it and for 17 children we had no information (17/48 35.42%) (figure 24).

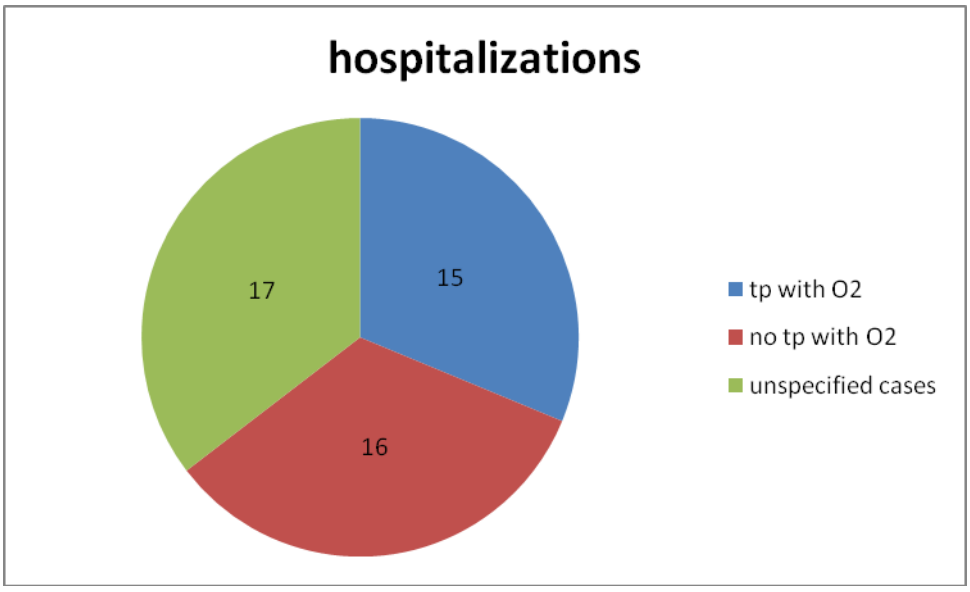


Figure 24. Distribution of pertussis hospitalizations according to the request of O_2 therapy.

Looking at the group of patients who had requested O_2 therapy, only 1 child was preterm (1/15 6.67%), 14 cases (14/15 93.33%) were born at term. In the group of patients who had not requested O_2 therapy, 2 case (2/16 12.5%) was preterm, 14 cases (14/16 87.50%) were born at term. According to these data, prematurity does not influence the severity of pertussis infection (Fisher's test P=1.0; OR=0.5; 95%CI from 0.04 to 6.17) (figure 25).

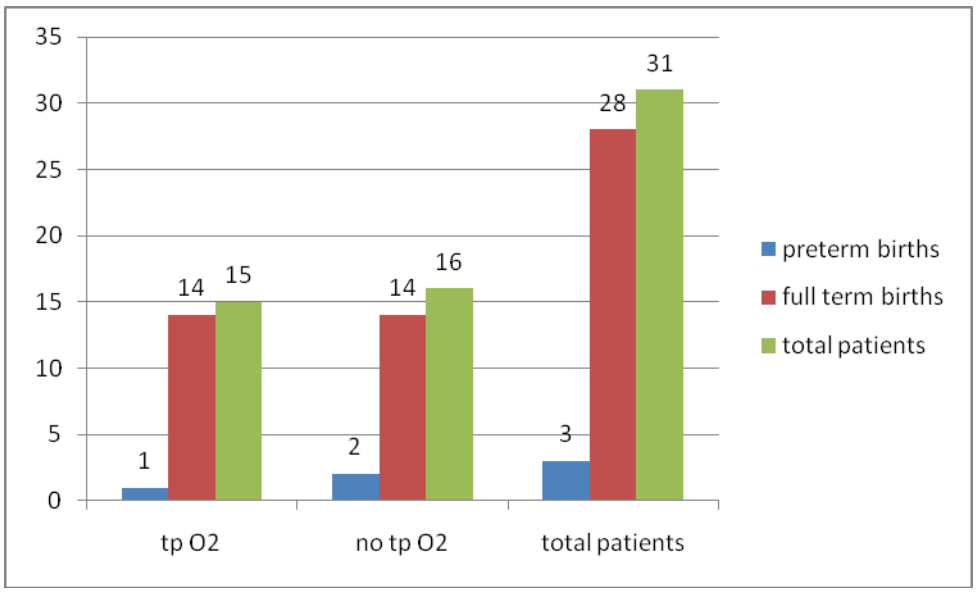


Figure 25. Distribution of pertussis cases according to treatment modalities divided into preterm and term patients.

Considering the vaccination status of the 15 patients who needed oxygen, 4 received at least 1 dose of the vaccine, while 11 received no dose. However, of the 16 patients who did not need oxygen, 6 received at least one dose of the vaccine, while 10 did not receive any dose. The difference between the two groups is not significant (Fisher's test P=0.704; OR=1.65; 95%CI from
0.36 to 7.60). Consequently, vaccination status is not a variable that influences the need for oxygen therapy (figure 26).

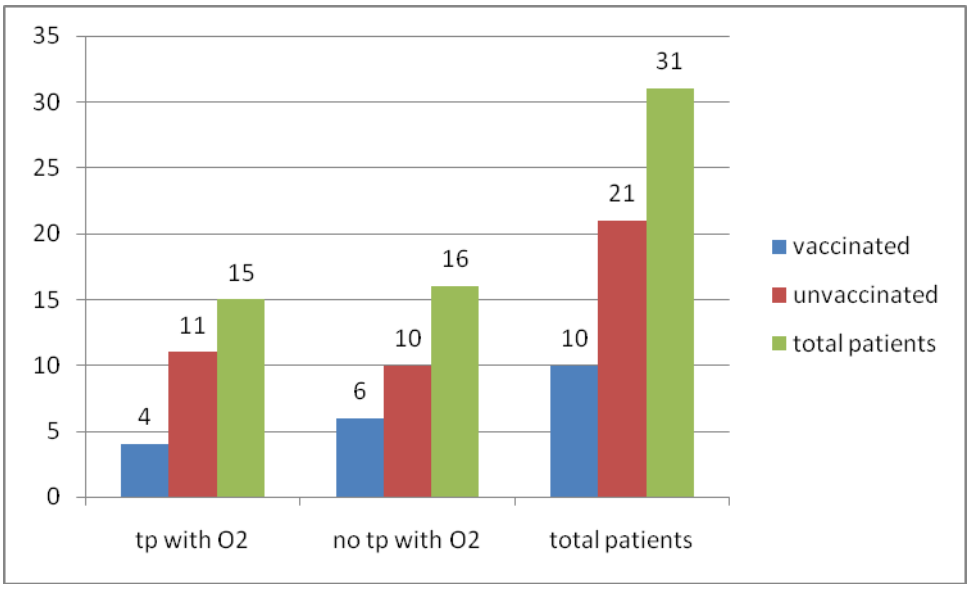


Figure 26. Distribution of pertussis cases according to treatment modalities, divided into vaccinated and non-vaccinated patients.

On the other hand, considering the age of onset of infection of 48 hospitalized patients, out of 15 children who had requested O_2 therapy (15/31 48.38%), 14 cases (14/15 93.33%) were less than one year old, 1 case (1/15 6.67%) was over the first year of life. In those who didn't need O_2 therapy (16), 12 cases (12/16 75%) had less than one year of life and 4 cases (4/16 25%) more than one year of life (figure 27). According to these results, the age of onset of disease is not a risk factor for the need for oxygen during pertussis infection (Fisher's test P=0.333; OR=4.66; 95%CI from 0.45 to 47.63).

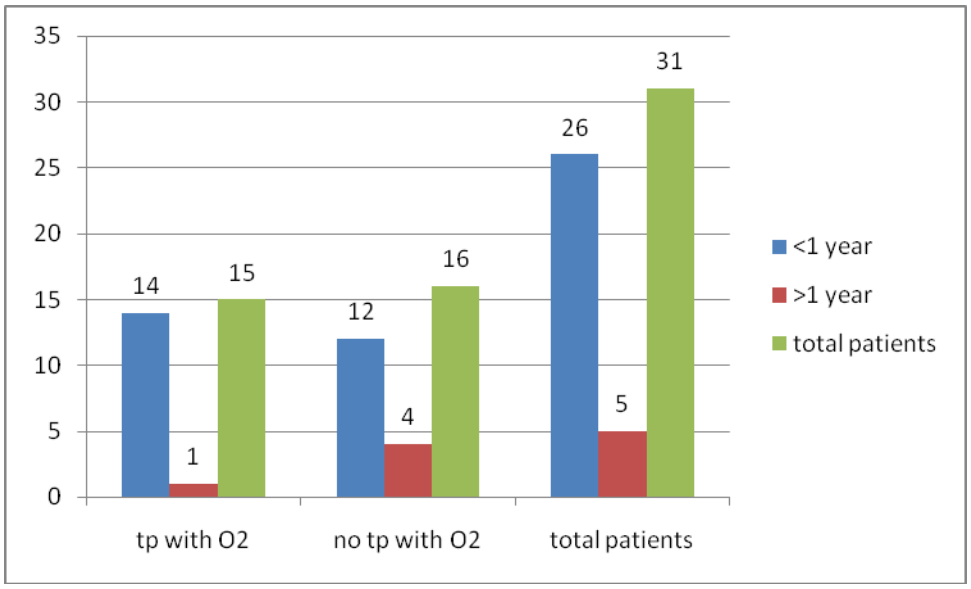


Figure 27. Distribution of cases of whooping cough by age, subdivided according to the modalities of care.

In the subgroup of 99 cases of whooping cough, I considered the 48 (48/99 48.48%) patients who requested hospitalization in a hospital ward and represented them according to the length of stay.(table 7)

CASES	REGION	PROVINCE	HOSPITAL	GENDER	DATE OF BIRTH	PREMATURITY	DAYS OF HOSPITALIZATION	OXYGEN THERAPY	VACCINE	VACCINE DOSES
1	Tuscany	Massa	Massa	f	28/04/2012	yes	6		no	
2	Tuscany	Florence	meyer	m	01/05/2012	yes	7		yes	2
3	Tuscany	Florence	osma	m	28/01/2013	no	12		no	
4	Tuscany	Florence	osma	m	28/01/2013	no	3		no	
5	Tuscany	Florence	empoli	f	05/04/2013	no	14		no	
6	Tuscany	Florence	meyer	f	30/10/2004	no	6		yes	3
7	Tuscany	Massa	massa	m	06/09/2013	yes	7		no	
8	Tuscany	Siena	le scotte	m	24/10/2013	no	15	yes	no	
9	Tuscany	Florence	empoli	f	18/01/2014	no	13		no	
10	Tuscany	Florence	meyer	f	08/09/2013	no	8	yes	no	
11	Tuscany	Florence	meyer	f	17/09/2008	no	24	yes	no	
12	Tuscany	Pistoia	pistoia	f	13/02/2014	no	6		no	
13	Tuscany	Florence	meyer	f	23/05/2014	no	6	no	no	
14	Tuscany	Florence	meyer	m	24/05/2014	no	5	no	no	

15	Tuscany	Florence	meyer	m	20/04/2014	no	5	yes	no	
16	Tuscany	Florence	meyer	m	25/05/2014	no	3	no	no	
17	Tuscany	Prato	prato	f	17/03/2014	no	3		yes	1
18	Tuscany	Florence	meyer	m	17/05/2014	no	4	no	yes	1
19	Tuscany	Florence	meyer	m	10/06/2014	no	4	no	yes	1
20	Tuscany	Florence	meyer	f	18/05/2014	no	4	yes	yes	1
21	Tuscany	Florence	meyer	m	11/08/2014	no	4	no	no	
22	Tuscany	Florence	meyer	f	02/06/2014	no	3	no	yes	1
23	Tuscany	Florence	meyer	f	06/11/2014	no	3	no	no	
24	Tuscany	Florence	meyer	f	27/07/2011	no	8	no	yes	3
25	Tuscany	Florence	meyer	f	06/02/2015	no	6	yes	no	
26	Tuscany	Florence	osma	f	17/12/2014	no	7		yes	1
27	Tuscany	prato	prato	f	14/03/2015	no	14	yes	yes	1
28	Tuscany	massa	massa	f	03/03/2015	no	10		yes	1
29	Tuscany	pisa	lotti	f	09/04/2015	no	9	yes	yes	1
30	Tuscany	Florence	meyer	m	02/05/2015	no	7	no	yes	1
31	Tuscany	prato	prato	f	19/08/2015	no	20	yes	no	
32	Tuscany	Florence	meyer	f	04/12/2012	no	6	no	yes	3
33	Tuscany	Florence	meyer	m	13/06/1997	yes	8	no	no	
34	Tuscany	Florence	meyer	m	17/10/2002	no	1	no	no	
35	Tuscany	Florence	meyer	m	27/11/2015	yes	7	yes	yes	2
36	Tuscany	Florence	meyer	m	25/05/2016	no	5		yes	1
37	Tuscany	Florence	empoli	m	17/08/2016	no	7	yes	no	
38	Tuscany	Florence	meyer	f	16/02/2017	no	10	yes	no	
39	Tuscany	Florence	osma	m	09/03/2017	no	7		no	
40	Tuscany	arezzo	arezzo	m	07/04/2017	no	21	yes	no	
41	Tuscany	Florence	meyer	f	15/04/2017	no	9	no	no	
42	Tuscany	Florence	meyer	m	20/03/2017	no	4		no	
43	Tuscany	Florence	meyer	m	03/02/2017	no	4		yes	1
44	Tuscany	Florence	meyer	f	14/04/2017	no	8	no	no	
45	Tuscany	Florence	osma	m	28/02/2017	no	5		no	
46	Tuscany	Florence	meyer	f	22/09/2015	no	15	yes	no	
47	Tuscany	Florence	meyer	m	25/07/2017	no	5	yes	no	
48	Tuscany	Florence	meyer	m	05/07/2017	yes	5	no	no	

Table 7.Characteristics of patients hospitalized for whooping cough.

The median of the days of hospitalization was 6 days. We used the median to give less weight in the calculation to the 24-day long hospitalization of a patient who was hospitalized not only for pertussis infection but also for chemotherapy.

Focusing on longer stays (>6 days) we found that these were 24.

However, among the children who needed a long hospitalization 4 were premature. Prematurity does not significantly affect the length of the hospitalisation (Fisher's test P=0.666; OR=2.20; 95%CI from 0.36 to 13.34). (figure 28)

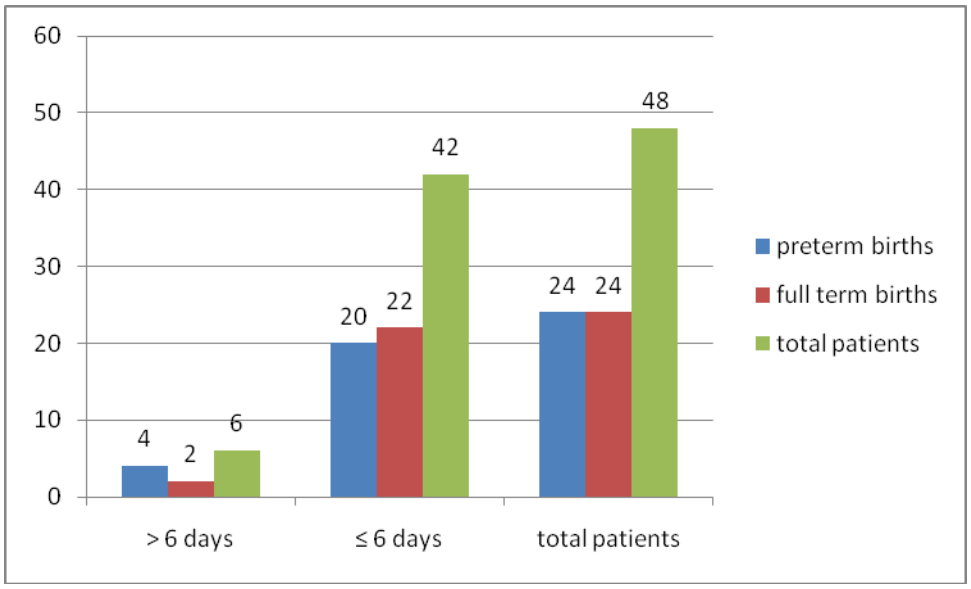


Figure 28. Distribution of cases of whooping cough by length of stay, divided into preterm and term patients.

In addition, the age of onset of the disease in this case does not significantly affect the length of hospitalization, in fact, according to our data, 3 children over the age of 1 year needed a long hospitalization and 3 children over the age of 1 year needed a hospitalization of normal duration (< or equal to 6 days). This is unexpected, but is probably explained by the fact that 4 " older " children (>1 year) had a comorbidity and therefore longer hospitalizations.(Fisher's test P=1.0; OR=1.00; 95%CI from 0.18 to 5.53) (figure 29)

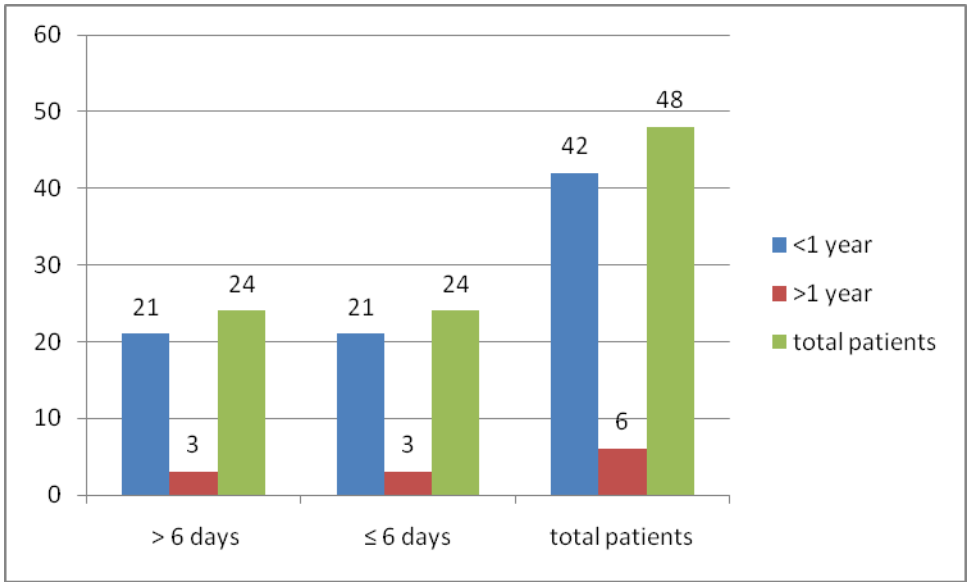


Figure 29. Distribution of cases of whooping cough by length of stay divided according to the age of onset of the infection.

Even having received the vaccination, whether complete or incomplete, or not, does not significantly affect the length of hospitalization. (Fisher's Test P=1.0000) (figure 30).

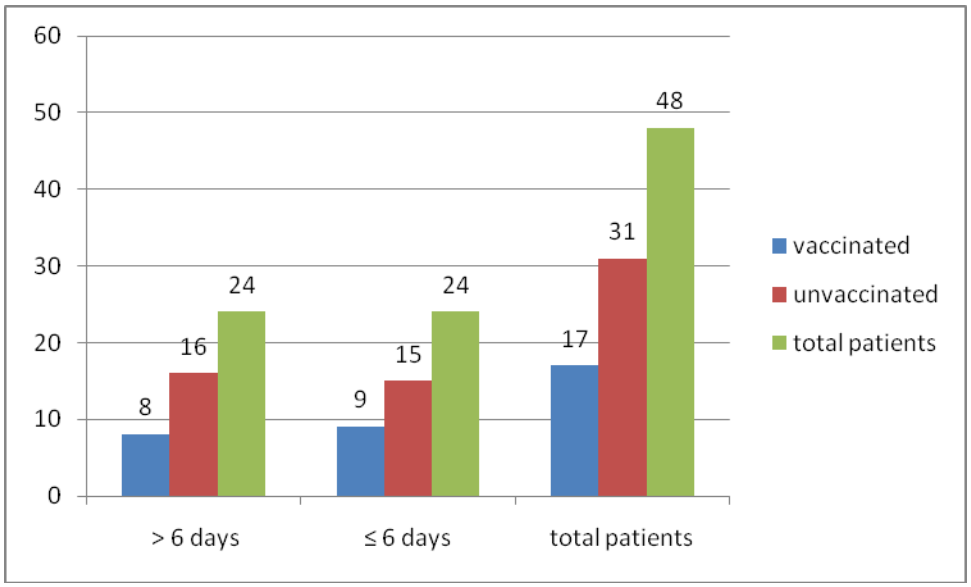


Figure 30. Distribution of cases of whooping cough by length of stay divided into vaccinated and non-vaccinated patients.

COMPARISON OF HOSPITALIZATION AND VACCINATION COSTS

In our sample of 51 patients admitted to the Meyer Children's Hospital due to whooping cough, we found that the median of the days of hospitalization was about 7 days.

The cost of one day's hospitalization was derived from the Annual Report on Hospitalisation Activities (SDO Data 2015) of September 2016 in use until January 2019, published by the Ministry of Health. We have obtained the cost of a daily hospitalization in public university hospitals for acute activities, in ordinary regime for the hospitalization of patients with infectious diseases. The cost is 121.10 EUR. Considering the number of patients admitted for pertussis (32) at the Meyer Children's Hospital, the total cost of hospitalization for the total number of patients in the sample is 46744.60 EUR. If we consider that the average duration of hospitalization calculated for our sample of patients is 7 days, the cost of hospitalization for infectious disease is 847.10 EUR for each patient.

The cost of administering a hexavalent vaccine containing the pertussis vaccine - such as GSK's Infanrix hexa, containing 125 µg filamentous hemagglutinin and 125 µg pertactin - is 44 EUR. The complete cycle of Infanrix to be completed at the 13th month with the 3rd dose, involves a cost per patient of 132 EUR. Of the 51 patients admitted to the Meyer Children's Hospital, those who did not comply with the vaccination schedule despite their adequate age for the practice of immunization were 15 born at term and 3 born at preterm, all unvaccinated. In addition there are 3 other patients not adequately vaccinated. One of them required one day of hospitalization, 4 patients 3 days of hospitalization, 2 patients 4 days of hospitalization, 4 patients 5 days of

hospitalization, 1 patients 6 days of hospitalization, 5 subjects 8 days of hospitalization, 1 patients

10 days of hospitalization, 1 patients 15 days of hospitalization and 1 case 24 days of hospitalization. If we calculate the total cost of hospitalization for infectious diseases of these 21 patients, taking into account the average length of hospital stay of 7 days, we can see that the costs of the hospital would amount to 17801.7 EUR total. If these 21 patients had carried out vaccination according to the vaccination schedule at the time of infection, the cost would have been € 1101.

We considered that 14 patients aged between 2 and 4 months should have received 1 dose of vaccine (44 EUR), a 7-month-old patient should have received 2 doses of vaccine (88

EUR), a 13- year-old unvaccinated patient should have received the full primary cycle (132 EUR) and a booster dose (15 EUR). A 18-years-old patient who had not received a vaccination dose in 12 years, should have received a booster dose (15 EUR). Another 5 year-old unvaccinated patient should have received the full primary cycle (132 EUR). Finally, 3 patients who have not been adequately vaccinated should receive: 2 of the second vaccine dose (44 EUR) and 1 of the booster vaccine doses (15 EUR). So if they had been appropriately vaccinated, the probability of a pertussis infection would have significantly decreased and consequently also the probability of hospitalization, providing the health system a saving of about 16700.70 EUR. (table 8)

hospitalization costs per day	total cost of hospitalization for 51 patients	Cost for 7 days hospitalization for each patient	costof vaccination	full cycle vaccination cost (3)	Inadequately vaccinated patients	hospitalization costs per day for 21 patients	total vaccine cost for 21 patients based on age of infection	Money saved
121.10 EUR	**46744.60 EUR**	**847.10 EUR**	**44 EUR**	**132 EUR**	**21**	**17801.70 EUR**	**1101 EUR**	**16700.70 EUR**

Table 8. Hospitalization costs of the hospitalized patients.

Of the hospitalized patients, 15 cases (15/51 29.41%) were under 61 days of age so they could not carry out any vaccination. If we calculate the cost of hospitalization of all 15 patients for The median length of hospital stay (7 days), the total cost would be 12715.5 EUR. If mothers of these 15 patients had been vaccinated in the third trimester of pregnancy with GSK Boostrix vaccine, the probability of pertussis in infants < 3 months would have been remote.

The GSK Boostrix vaccine contains the vaccination against dTp at lower dosages such as 8 μg of pertactin and 8 μg of filamentous hemagglutinin as it is used as a booster and its cost

is 15 EUR. The total cost of vaccination of 15 mothers would have been 225 EUR and therefore the saving would have been 12490.5 EUR. (table 9)

patients < 3 months	cost of hospitalization stay 7 days for 15 patients	total vaccine booster cost in pregnancy
15	12715.5 EUR	225 EUR

Table 9. Savings that would have benefited if the mothers of hospitalized patients had been vaccinated.

If the unvaccinated patients had been vaccinated and the mothers of those patients who could not access the vaccination because of age, and assuming that the vaccine had protected the patients from infection, we could have saved 29191.2 EUR.

EXPERIMENTAL RESULTS

Of the 194 *B. pertussis*-positive samples that were collected in 2010–2019, 71 underwent molecular characterization. These samples were randomly selected from the 194 total samples.The remaining 123 samples were not available.

Three virulence-associated gene, ptxP, ptxA and prn,were analyzed using PCR-based sequencing. ptxA and prn are proteins used in ACVs.

Among the 71 *B. pertussis* strains tested, 3 ptxP (ptxP1, ptxP3 and unidentifed ptxP), 1 ptxA (ptxA1), 2 prn (prn2 and prn1) alleles were identified.

ptxA alleles

The ACVs currently used in the European countries contain ptxA2 and ptxA4 [224]. The non-vaccine type allele ptxA1 was identified in all *Bordetella pertussis* positive samples analysed (71/71 100%).

Multiple sequence aligneament of all ptxA1 sequences (from 474 to 1350 position) presented homogeneity and an 100% affinity to the ptxA1 taken as reference (GenBank reference: AJ245366.1). (figure 31)

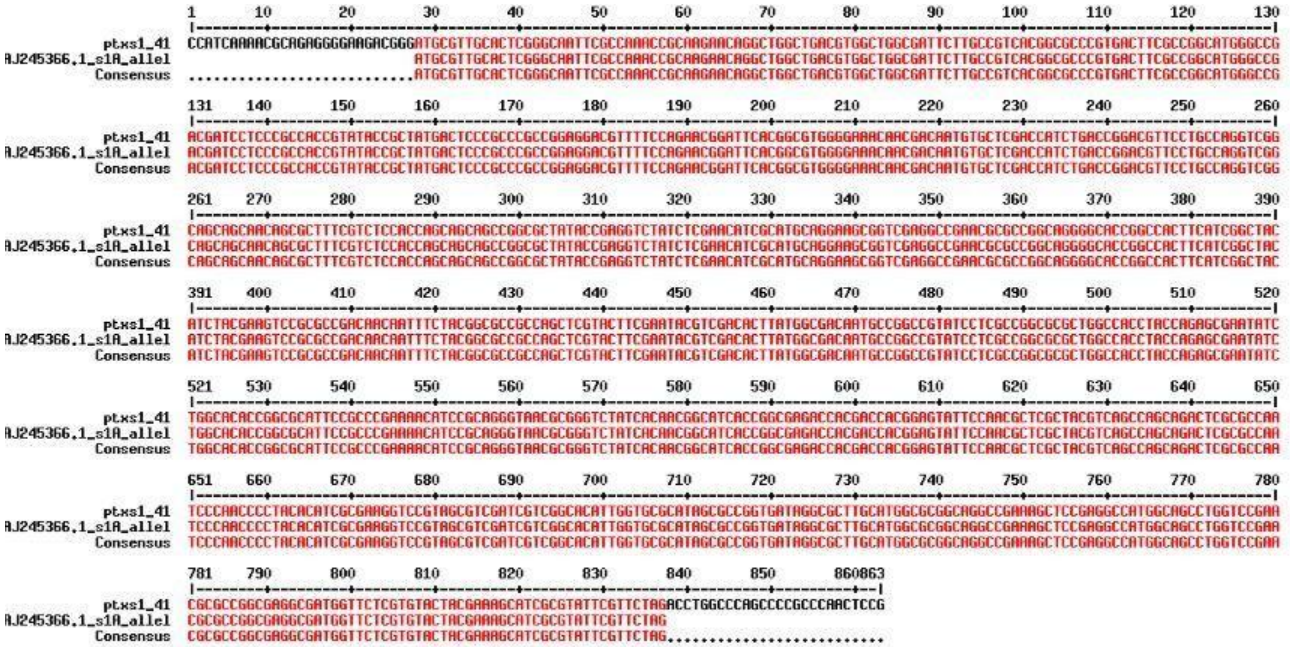


Figure 31. Alignmet between a ptxA1 sequence of sample group and a ptxA1 reference sequence.

Prn alleles

The ACVs currently used in the European countries contain prn1 and prn 7 [224]. Prn gene structure suggestes that polymorphism may occur in two regions, desinated regions 1 and 2, that are comprised of repeats coding for the amino acid sequenze GGxxP and PQP, respectly .The prn region1 (from 649 to 1234 position) was sequenced in 43 samples of the 71 total (43/71 60.56%). 42 of these 43 samples presented prn2 allele (figure 32), while the remaining one sample had prn1/7 allele (figure 33). Subsequent sequencing of the variable region 2 of prn was not performed, to distinguish between prn1 and prn7 allele. The simultaneous alignment of prn sequenze of our sample group showed a high homogeneity and high positive score that measure the similarity compared the prn2 sequence taken as reference. Only one sequence of prn presented only one single point mutation (SNP) compared to other sequences, and it corresponded to the allelic form prn1.

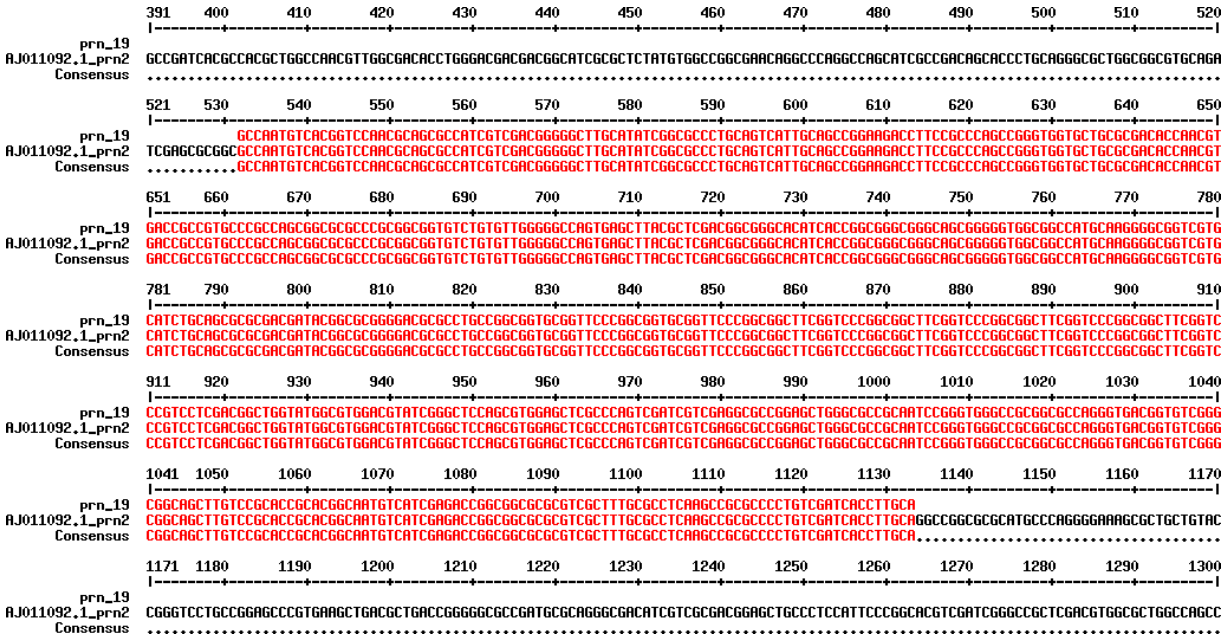


Figure 32. Alignmet between a prn2 sequence of sample group and a prn2 reference sequence (GenBank reference: AJ011092.1).

Figure 33. Alignmet between a prn1/7 sequence of sample group and a prn1 and a prn7 reference sequences (GenBank reference: AJ011091.1, AJ133784.1).

ptxP alleles

The pertussis toxin promoter, ptxP, was also included, as several previous studies have shown that the ptxP3 allele is an important characteristic of recent clinical isolates [222] and ptxP3 strains have gradually replaced the predominant ptxP1 strains in recent years. ptxP3 strains produce more Ptx than ptxP1 strains and as a result they are more virulent than the ptxP1 strains. ptxP3 allele, possess the base “A” in the ptxA promoter region in position -65 relative to the transcriptional start site [225]. ptxP1 allele contain the base “G” in this position.

69 of the 71 samples presented at the ptxP3 allele (69/71 97,18%) (figure 34). The remaining two samples had: one ptxP1 allele (1/71 1,41%) (figure 35) and one had an unidentified ptxP (1/71 1,41%) allele (figure 36). The sequence of the unidentified ptxP allele was identical to ptxP3 allele except for one single point mutation (SNP).

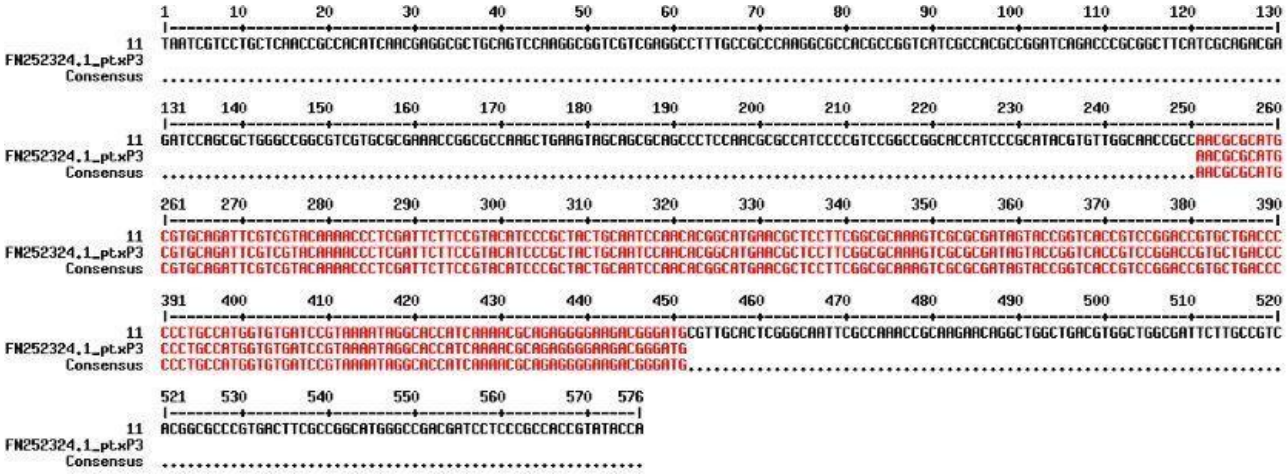


Figure 34. Alignmet between a ptxP3 sequence of sample group and a ptxP3 reference sequence (GenBank reference: FN25324.1).

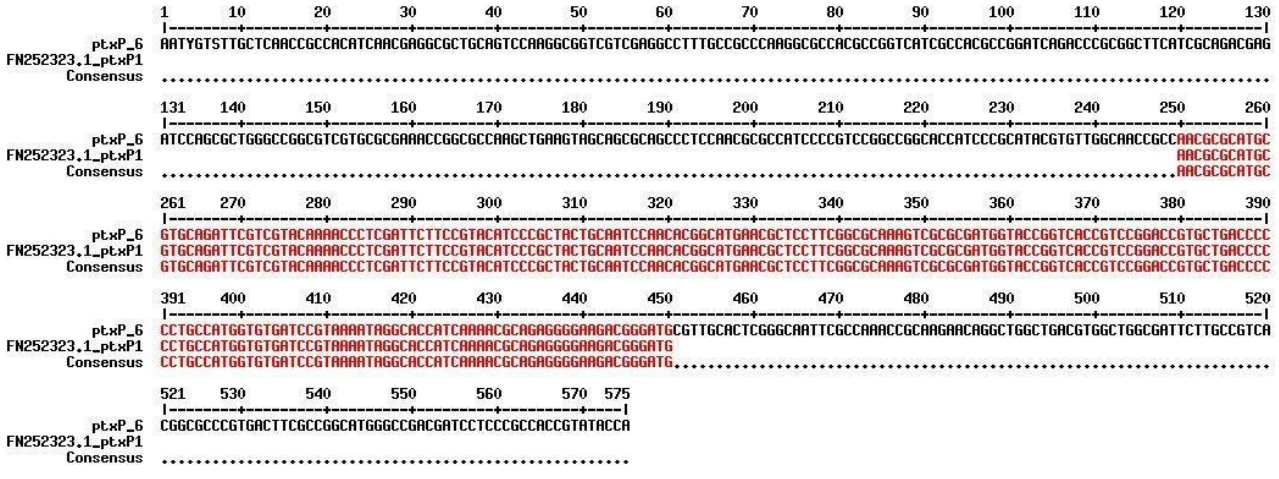


Figure 35. Alignmet between a ptxP1 sequence of sample group and a ptxP1 reference sequence (GenBank reference: FN252323.1).

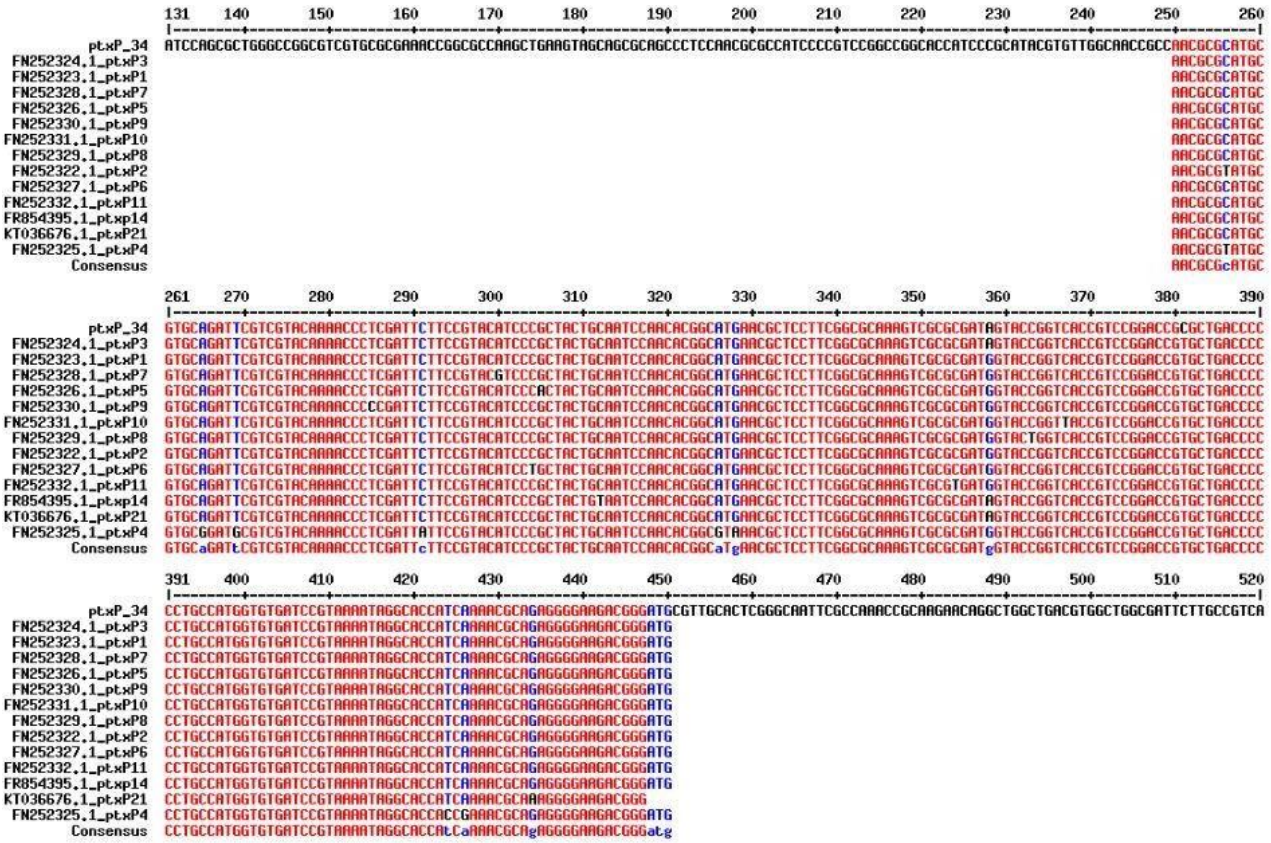


Figure 36. Alignmet between a unidentified ptxP sequence of sample group and ptxP alleles reference sequences found in GenBank (GenBank reference: FN252323.1, FN252322.1, FN252325.1, FN252326.1, FN252327.1, FN252328.1, FN252329.1, FN252330.1, FN252331.1, FN252332.1, FN25324.1, KT036676.1, FR854395.1).

B.pertussis strains profile

The prn region1 was sequenced in 43 samples of the 71 total (43/71 60.56%). In 42 of these 43 samples (42/43 97.67%), the allelic profile was ptxA1-ptxP3-prn2. The remaining one sample had ptxA1-ptxP1-prn1/7 profile (1/43 2.32%). In the other samples where only the ptxA and ptxP genes were sequenced (28/71 39,44%), the allelic profile was: in 27 samples of the 28 total ptxA1-ptxP3, while in only one the allelic profile was ptxA1-unidentified ptxP.

DISCUSSION

Whooping cough is a highly contagious bacterial infectious disease. This study aims at the epidemiological analysis of the infection and its major risk factors, to obtain useful indications regarding prevention. In addition, my study aims at studying the strains of *B. pertussis* that circulate in Tuscany. In fact, we want to understand if in Tuscany, as in most of Europe, new varieties of *B. pertussis* are emerging.

Our study population included subjects who had symptoms compatible with pertussis infection and who had been molecularly diagnosed at Meyer Children's Hospital's Immunology Laboratory between 2010 and 2019.

Through the collected data, we have seen that, the number of cases of whooping cough, which were obtained through the Register of Invasive Bacterial Diseases of the Immunology Laboratory of Meyer Children's Hospital, increased from 2010 to 2016 by about 10 times in parallel with a decrease in vaccination coverage during the same period, in fact in 2010 the vaccination coverage for whooping cough was 96.4% reduced to 94.6% in 2014 until 93.5% in 2016.

It is important to underline that the incidence data are underestimated, as the data collected by the Immunology Laboratory of the Meyer Children's Hospital are just a part of all cases of whooping cough in Tuscany. However, we can speculate that the the increasing number of cases from 2010 to 2016 may be related to the progressive reduction of vaccination coverage for whooping cough in the same period.

These results make us understand that the effectiveness of anti-pertussis vaccination is reduced when there is a reduction in vaccination coverage. In this period characterized by a fearsome and widespread *vaccine hesitancy*, coverage tends to decline for all diseases preventable by vaccination. This phenomenon leads subjects, who by age cannot yet be vaccinated or immunodeficient subjects, to lose the benefit of herd immunity. The situation is most evident in whooping cough, where the most affected group is that of infants.

In favor of these statements, the latest updates provided by the Institute of Health, showed an increasing trend in vaccination coverage from 2017 to 2018. In 2018, vaccination coverage was estimated at 95.07%. At the same time, the number of patients with

whooping cough has fallen dramatically.

We have analyzed the age distribution of our population, showing that the majority of cases are children under one year of age, with 119 cases out of 194 of the total sample. In particular, more than a third of infections occur in the first 60 days of life. Given that the hexavalent vaccine from the national vaccination calendar is carried out from the 61st day of life, this large infant population cannot benefit from this type of active immunity and therefore when the herd immunity is reduced the cases of whooping cough increase. In this age group we have also characterized the course of pertussis infection according to age, observing that almost all hospitalized patients are subjects under one year of age. In particular, among hospitalized subjects, children under the age of one year are those who were most likely to have a pertussis infection that required oxygen therapy, although the case history requires a greater number of patients for it to be reliable. So we can conclude that not only does a child under one year of age fall ill much more, but he is also more likely to have a more serious infection. As far as the duration of hospitalization is concerned, it does not seem to depend on the age below one year of patients. However, this result could be influenced by the fact that in our case study, the subjects with longer hospitalization stays were subjects older than one year, all with comorbidity. Our data, are in agreement with those present in the literature [227], and highlight how, with regard to pertussis infection, the greatest impact in medical and economic terms is represented by the paediatric population under one year of life. The efforts of the scientific community and health prevention policy must be directed primarily towards implementing a vaccination strategy that can reduce the incidence in this particularly vulnerable age group. Particularly for children under 60 days of age, who cannot receive hexavalent vaccination, the importance of indirect prevention is stressed. In particular, indirect prevention can be achieved by reducing the number of susceptible individuals, thus reducing the spread of the pathogen within the population and thus the incidence of infection even in the categories of individuals who cannot receive the vaccine.

This reduction in cases can be achieved by reaching high rates of vaccination coverage for whooping cough (herd immunity) or limited to the community of the child, through the vaccination of all family members who care for the infant in the first months of life (*cocoon strategy*). Unfortunately, in the current historical moment, infants cannot benefit from high rates of vaccination coverage, and the cocoon strategy alone, from the data available in the

literature, does not seem to be an effective method to reduce the incidence in children under six months of age. At the same time, the cocoon strategy is an not very economically sustainable strategy. The best strategy therefore appears to be that which aims at providing the infant, in the first months of life, passive immunity through the transplacental passage of specific anti-pertoxic antibodies from the mother to the fetus. Previous studies have shown that to optimize this translplacental antibody step it is important to vaccinate the mother during the third trimester of pregnancy, regardless of the interval between doses [190].

We have also analysed from a pharmaco-economic point of view the sustainability of this vaccination strategy, demonstrating a gain in economic terms, for children who were less than 60 days old and who were hospitalised: if their mothers had received the vaccine, the passage of transplacental immunoglobulins during the last months of pregnancy would have guaranteed them passive immunity, so they would probably not have fallen ill and would not have been hospitalized (as evidenced by previous studies) [236]. In favor of this consideration, to limit *B. pertussis* infections and protect unvaccinated infants, the new approved National Plan of Vaccine Immunization recommend that the mother should be immunized during the third trimester of pregnancy [228, 229]. Unfortunately, until now, in Italy there was no implementation of this strategy and the pregnant women are not correctly informed or not informed at all about the possibility to get the pertussis vaccine.

However, unfortunately, in our subgroup population of 99 subjects, there are also 29 adequately vaccinated subjects who have been infected with *Bordetella pertussis*. Analyzing the period between the date of collection of the positive pharyngeal swab for Bordetella pertussis, and the date of the last vaccination, we observed a average time interval of about 4 and a half years. Therefore, in the light of these results, we have considered that this time interval is the average time of loss of vaccination efficacy after which patients may return being susceptible to infection.

Moreover, out of 45 patients who had received an incomplete or full primary cycle, one third of the cases had performed one of the three overdue doses, and they presented the infection during this delay period. For this reasons, in order to increase the effectiveness of the vaccine, it is important to strictly adhere to the indications of the national vaccination

calendar as regards both the primary cycle and subsequent booster doses.

Although the anti-pertussis vaccination is not effective in any subject, our results show that those who received the primary vaccination cycle (21/99 21.21%), regardless of the distance between the last booster and the time of onset of the disease, do not develop a severe infection, that is, requiring hospitalization. In fact, the only three fully vaccinated cases (3/31 14.29%) that required hospitalization had a comorbidity that could explain it. In addition, our data show that even receiving at least one dose of the vaccine can reduce the likelihood of having a severe infection that requires hospitalization. Therefore it is demonstrated that the vaccination effectively reduces the rates of hospitalization, as was also shown by the analysis on the sample of 194 subjects (Table 5) Reducing the rates of hospitalization means significantly reducing the costs of public health, as demonstrated by our pharmaco-economic analysis.

Finally, we also focused our attention on the group of preterm births. In fact, these children, depending on the region in which they are born, currently receive 3 or 4 doses of hexavalent vaccine. This last vaccination strategy has been adopted because some studies in the literature seem to show a lower level of immunogenicity of the hexavalent vaccine in preterm births compared to term births [175,176]. In Tuscany in the last ten years is in force the calendar with three doses for both full term and preterm births.

The incidence of prematurity in our sample of subjects is equal to that of the general population in the group of sick subjects. None of my studies shows a strong correlation between prematurity and hospitalization and between prematurity and aggravation and duration of hospitalization. Therefore, on the basis of our results, it can be concluded that prematurity is not a particular risk factor for whooping cough infection, nor is it a determining factor for its severity.

If the immunogenicity of the vaccine in preterm births had really been lower than in term births, we would have observed a much higher number of infections among preterm births. Moreover, our clinical practice study does not show differences in vaccine effectiveness between the two populations (infants born at term and infants born at term). That is why we believe that the three-dose vaccination schedule can be equally effective in both groups.

Studies on cohorts larger than ours will be necessary to confirm our data. However, we can conclude that the three-dose schedule pertussis vaccination is effective for both full term and preterm births, which unfortunately does not guarantee perennial immunity but which has a very high efficacy in reducing cases of complicated pertussis, which are the cases that weigh most heavily from a medical and economic point of view.

Despite the high vaccination coverage level, Europe has recently experienced an apparent resurgence of pertussis. In our study, *B. pertussis* strains circulating in Tuscany were subjected to molecular characterization order to understand if in Tuscany, as in most of Europe, new varieties of *B. pertussis* are emerging : of the 43 samples investigated for three virulence-associated gene, ptxP, ptxA and prn, all but 1, had the variant ptxA1-ptxP3-prn2 allelic profile. In the current acellular pertussis vaccines these antigenic alleles are not present, since the allelic profile of vaccinal strains is *prn1-ptxA2-ptxP1-fim3A.* Between the remaining 28 samples in which we have studied only two antigens, 27 samples had the variant ptxA1-ptxP3 allelic profile and one the variant ptxA1- unidentified ptxP allelic profile. The patients from whom these samples were obtained belonged to age group 0-18 years. The hypervirulent ptxA1-ptxP3-prn2 variant now circulates in many other European countries apart from Italy [222, 230]. Notably, these European countries use the acellular vaccine [231]. By contrast, Poland, which uses a whole cell vaccine, shows a different predominant *B.Pertussis* profile, namely, ptxA1-ptxP1-prn1 (allele profile present only in a single sample of our study). This is supported by a study in China, where the whole cell vaccine is used too: the predominant *B.Pertussis* allelic variant in China differs from the predominant strain that circulates in countries that use acellular vaccine. Such data suggest that the acellular and whole cell vaccines may select different *B. pertussis* populations [231, 232]. The antigenic divergence observed between vaccine strains and circulating strains in Europe may act synergistically with the ptxP3 polymorphism by enhancing transmission by hosts primed by vaccination. Pertussis among recently vaccinated children is rare, indicating that pathogen adaptation does not play a role unless immunity has waned. Many studies propose that waning immunity and pathogen adaptation have contributed to the resurgence of pertussis, although other factors such as increased awareness and improved diagnostics have also played a role [222, 233]. The effect of pathogen adaptation on disease impact may depend on factors such as vaccine coverage and the quality of the vaccine used. Although the incidence of pertussis in Italy decreased after the introduction of

immunization [234], it was seen that the disease has resurged in recent years. It seems unlikely that this resurgence is only due to the slight decline in vaccination coverage that was observed in 2014-2016. It is much more likely that the most important cause explaining the resurgence are the loss of vaccine efficacy and the changes in circulating B. pertussis strains [235].

In order to try to develop a new pertussis vaccine several different strategies are currently being explored. They include the use of different adjuvants, the addition of novel antigens, new formulations in microparticles or outer membrane vesicles and there is also a live attenuated pertussis vaccine candidate that has reached clinical development. However, the development of new pertussis vaccines takes time and is hampered by several important hurdles that have to be overcome. Meanwhile, an optimized use of the currently available vaccines may have a positive impact.

CONCLUSION

Our study results confirm that in Tuscany the decrease in vaccination coverage corresponds to a significant increase in cases of whooping cough. The most affected subjects are the youngest children, especially those under the age of one year, and infants in the first three months of life. Therefore, any effort aimed at increasing awareness of the practice of vaccination in pregnancy, which is currently the only effective method to reduce pertussis among infants under 3 months of age, is essential. These subjects are not only those who have the greatest risk of getting sick, but also have the greatest risk of presenting with severe infections that require hospitalization. The pharmaco-economic analysis of the costs appears to be very much in favour of this practice. With regard to preterm patients, the available data from the literature are not confirmed [179, 180, 183, 184, 189]: in fact, according to our results, the incidence of pertussis in preterm subjects is not greater than the infection in term subjects, since the proportion of preterm subjects in our sample is not greater than the general population. These data derive from a period of time in which a three-dose vaccination schedule was carried out in Tuscany, so according to our data the fourth vaccination dose during the primary cycle carried out in some regions of Italy does not appear necessary. Unfortunately, our study confirms that vaccinated subjects do not have perennial immunity, and that after about 4 years there is a progressive loss of coverage, with an increase in cases. This information is particularly important because it shows us that vaccination efficacy is likely to be reduced more quickly than the deadlines set for booster vaccinations. Our work, in line with other studies in the literature, absolutely emphasizes the need to optimize and standardize the vaccination schedule for term and preterm births, and to increase the pertussic vaccination for each pregnancy.

Moreover, the findings of the present study suggest that enhanced surveillance of pertussis and systematic laboratory confirmation of cases are needed in Italy. In order to assess the real epidemiology of the disease and the emergence of new strains that can escape the immunity induced by vaccination. Consequently, it would be necessary to verify whether the current commercial vaccine used is effective against new circulating strains with an allele structure different from that of the vaccine antigens (ptxA2/4-prn1-ptxP1). It will be

interesting to extend our study to the sequencing of the other two vaccinal antigens fha and fim 2/3 in order to identify which allele variant is currently circulating for these two antigens. We can therefore conclude that monitoring of strains in circulation is also crucial for designing future national vaccination strategies and for informing research into new pertussis vaccines.

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