SAPIENZA
UNIVERSITÀ DI ROMA

FACOLTÀ DI MEDICINA E ODONTOIATRIA

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DOTTORATO DI RICERCA

TECNOLOGIE BIOMEDICHE INNOVATIVE IN MEDICINA CLINICA

XXIX CICLO

WHY PERTUSSIS IS STILL A CONCERN?
REASONS FOR RESURGENCE AND IMPROVING STRATEGIES

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ABSTRACT

Pertussis is a highly contagious, vaccine-preventable, bacterial infectious disease, caused by *B. pertussis*. It affects children less than 6 months of age with severe clinical symptoms especially in newborns. Pertussis continues as a public health concern threat given its re-emergence despite high vaccination coverage; re-emergence could be caused by the waning of vaccine immunity consequent to introduction of the acellular (aP) vaccine, by the adaptation of circulation of variants *B. pertussis* strains and by the improvement of diagnostic methods and active surveillance. We conducted a longitudinal case-controls double center study on 141 infants younger than 6 months, hospitalized with typical symptoms of pertussis and 235 healthy controls. Inclusion criteria: age lower than 180 days, with cough lasting more than 5 days, paroxysmal cough, apnea or cyanosis and post-cough vomit. Exclusion criteria: chronic diseases and genetic syndromes. A total of 157 breastfeeding mothers from cases and controls infants were included in the immunological study and requested to undergo a blood and breast milk sample collection. A total of 167 parents from 145 infants were enrolled in the study about the serum-epidemiology of pertussis.

Our project leads to identify a prevalence of more than 50% of pertussis cases among the population studied. We demonstrated that breastfeeding does not exert a protective role against pertussis infection in infants, showing a low immunologic activity of breast milk against *B. pertussis*, compared with other pathogens, and that the presence of at least one sibling doubled the risk to contract pertussis. In addition, comparing the specific immune response of mother of cases and mothers of controls, we showed that both mothers groups have pre-existing pertussis-specific antibodies and memory B cells and react against the infection with a recall response increasing the levels of specific serum IgG and the frequency of all isotypes of memory B cells. Finally, we found that 40% of parents of infants hospitalized with pertussis had a serological evidence of recent infection, and only 30% showed respiratory symptoms in the previous 3 weeks; interestingly, 30% of parents who not reported symptoms had a serological evidence of recent infection. The combination of these results allows to suppose a source of contagion in almost 90% of them.

In conclusion, the prevalence and severity of the disease among infants should increase the attention on the disease, leading to better strategies for its prevention and care. It is mandatory to advise parents about pertussis transmission to the newborns and to be aware of respiratory symptoms in the household. Breastfeeding remains a milestone of prevention for several infectious diseases, but in our study, we showed no protective role in the prevention of *B. pertussis* infection. Vaccination remains the major strategy for the prevention of pertussis but, this project supports an improving in pertussis surveillance, the detection of escape mutants, and the development of more effective vaccine.

1. INTRODUCTION

Pertussis (or whooping cough) is a highly contagious infectious disease, caused by a Gram-negative bacterium Bordetella pertussis (*B. pertussis*) [1-4], and is a vaccine-preventable disease. The clinical symptoms of pertussis change with age, previous exposure to *B. pertussis* and immunization status. It primarily affects children less than 6 months of age with severe clinical symptoms especially in newborns, causing an excess of admissions to intensive care units [5-6]. Most infants have a typical paroxysmal cough which can last more than two months [7], bronchitis, with complications including pneumonia, seizures, encephalopathy, and possibly sudden infant death [5]. Pertussis is particularly severe during the first year of life, while adolescents and adults may have a mild course often with an atypical clinical picture with prolonged cough [8].

Infants pay the highest price in terms of morbidity and mortality for pertussis [9], worldwide and especially in developing countries. Pertussis continues as a public health concern threat given its re-emergence despite high vaccination coverage [1,10,11]. Epidemic cycles reoccur every 2 to 5 years and 2015 has witnessed the worst outbreak in the past 70 years [9]. Pertussis disease re-emergence is due to many factors; several authors recognized in the waning of vaccine immunity consequent to introduction of the acellular (aP) vaccine, in the adaptation of circulation of variants *B. pertussis* strains (different from the vaccine strains) and the improvement of diagnostic methods and active surveillance due to increased awareness the possible determinants of this issue [12-15].

Italian vaccinal schedule provides for the administration of three doses of the vaccine against pertussis in the first year of life (at 2-3 months, 5 months and 11 months). Moreover, are provided boosters at 6 and 12 years of age, and every ten years after the 18th year. Several authors demonstrated that while partially immunized infants may experience a milder clinical course of the disease, pertussis can be life threatening in unvaccinated infants [9].

It is demonstrated that the circulation of *B. pertussis* among households is a major source of the transmission of the disease to infants too young to be vaccinated [16,17]. Thus, alternative prevention strategies have been proposed for protecting very young infants, including vaccination in newborns, cocooning protection [17,18] and one of the keys to address prevention strategies for infant in the first months after birth is to focus on the crucial role of a pertussis maternal immunization during pregnancy [18-20]. In Italy, the concept of household transmission is not widespread among parents and there is a lack of awareness that pertussis may cause severe or chronic cough in adolescents and adults.

Several reports demonstrated that breastfeeding protects the newborns from several respiratory and gastro-enteric pathogens, as demonstrated by the observation that the mortality rate due to infection is reduced by half in neonates receiving maternal milk [21].

Exclusive breastfeeding is demonstrated to reduce the risk of upper respiratory infections [22] lower respiratory tract infections and pneumonia, in terms of hospitalizations and mortality [23]. The role of breastfeeding in protecting from *B. pertussis* has not been well-characterized yet. Although it has been demonstrated that breast milk contains antibodies against *B. pertussis* few data exist on the actual protective activity of breast milk against pertussis from an epidemiologic perspective.

1.1. Epidemiology

Currently, there is no global epidemiological data on pertussis. Challenges in estimating the global pertussis disease burden are linked to different factors. First, there are limited surveillance systems established in many countries, with few resources being allocated to improve the coverage and accuracy of these systems. The lack of surveillance systems impacts the timely collection of data and leads to underreporting the number of cases. High-income countries (HIC) typically have a higher number of reported cases globally, with minimal cases reported in low-to-middle-income countries (LMIC). In LMIC, incidence and attributable mortality rate data can be problematic to obtain accurately due to poor infrastructure and a lack of coverage in civil registration systems, which not provide an accurate picture of the burden of pertussis disease.

The second challenge is access to adequate laboratory infrastructure and pertussis tests, especially LMIC; it is estimated that almost half of LMIC populations live in rural areas [10, 24], where access to health systems, the specimen collection and lengthy transport to laboratory centers, proves to be a significant challenge [25].

Despite high vaccination coverage, the disease is endemic worldwide and epidemic cycles are still occurring every 2–5 years [9,11]. The World Health Organization estimates that in 2019, there were 132754 cases of pertussis globally. The annual worldwide incidence of pertussis is estimated to be 48.5 million cases, with a mortality rate of nearly 295,000 deaths per year. The case-fatality rate among infants in low-income countries may be as high as 4%. [26]. The incidence rate of pertussis has significantly decreased since introduction of the pertussis vaccine. Nevertheless, during the last decades, the incidence of reported cases has increased, especially in adolescents and adults, even in countries with a high vaccine coverage [9].

For 2017, 29 EU/EEA countries reported 42242 pertussis cases. Five countries (Germany, the Netherlands, Poland, Spain and the UK) accounted for 76% of all notified cases. European notification rate was 9.4 per 100000 population.

In the countries reporting the highest notification rates (Norway, the Netherlands, Germany and Denmark), adults (≥18 years of age) accounted for a large proportion of cases, highlighting the fact that pertussis is not only a pediatric disease. Information on age was available for 48095 cases

(99.7%); 47% of cases were aged 30 years or older and an additional 15% were in the age group 15 to 29 years. A total of 62% of cases were above the age of 14 years.

In the pediatric age group, the highest notification rate was observed among infants below the age of one year (53.9 cases per 100000 population). The highest rates in infants were reported in Luxembourg (146.9 cases) and Austria (145.1), followed by Denmark (138.7) and Ireland (126.8). Among infants with known age in months (90%), 70% were < 6 months of age and 50% were < 3 months of age. A second peak was observed in 10–14-year-olds (25.0 cases per 100000 population) children [27].

Deaths from pertussis disease are recorded every year in Europe, often are among infants who were too young to have been fully vaccinated. Less than one child in every thousand will die due to pertussis, however in many instances pertussis is not recognized as the cause of death, so it is possible that pertussis is responsible for a higher number of deaths than actually recorded. Almost all deaths recorded in Europe are in infants younger than three months [9,28].

Globally 85% of the target population is covered by the DTP3 vaccine protecting against pertussis. By 2018, 129 countries had reached at least 90% coverage of DTP3 vaccine [9-26]. In Italy, pertussis vaccination coverage is estimated to be around 95% and is offered free of charge, included in the hexavalent vaccine, in combination with other vaccines against diphtheria, tetanus, polio, hepatitis b and Haemophilus influenzae type b [28].

1.2. Microbiology and Pathogenesis

Pertussis pathogenesis is mediated by many virulence factors, such as toxins and surface molecules, which are under the control of a single genetic locus: Bordetella Virulence Regulon AS (bvgAS) [5, 6, 29].

Bordetella species belong to Gram-negative bacilli [6, 30]. Six species of Bordetella have been identified: *B. pertussis, B. parapertussis, B. bronchiseptica, B. avium, B. holmesii and B. hinzii*. The most important pathogenic species for humans is *B. pertussis. B. parapertussis* causes a disease very similar to whooping cough in humans, but generally milder. On the other hand, *B. bronchiseptica* is pathogenic for some species of domestic animals (dogs, cats…).

Different species share many genotypic characteristics; for example, *B. pertussis* and *B. parapertussis* differ because *B. parapertussis* does not express the gene encoding the pertussis toxin (resulting in a lower virulence) [11].

B. pertussis is a slow-growing microorganism and generally, it takes 7-10 days to grow, isolate and identify the organism; these very long times are a clear limit to the use of culture examination for the diagnosis of each individual case.

This microorganism produces a wide range of toxins and biologically active substances that play an essential role in pathogenesis and immunity [17] (Figure 1).

The main *B. pertussis* virulence factor is pertussis toxin (PT), composed by an oligomer (subunit B) that binds the target cells and a protomer (subunit A) with ADP-ribosylation action, which acts at the level of a G protein of membrane regulating adenylate cyclase (AC). This induces an increase in c-AMP and causes hypersecretion of bronchial mucus. PT has also an inhibitory activity against lymphocyte migration at the bronchial level and contributes to the bacterial adhesion to the bronchial ciliated cells [17,30]. The novel allele ptxP3 of the PT promoter is replacing almost completely the classic allele ptxP1 and it has been related to epidemics in Europe, Asia, US and Australia in the last years. The ptxP3 strain is more virulent and produces higher levels of PT, and is associated with severe pertussis cases and with an increased incidence of hospitalization and deaths. Furthermore, ptxP3 strains have a selective advantage, because they are genetically different from the ones contained in the current vaccines [31-33].

Filamentous Haemagglutinin (FHA) plays an important role in the adhesion of *B. pertussis* to host cells [5]. Some studies have shown that there is an interaction between receptors on macrophages and FHA, which results in the suppression of the synthesis of the pro-inflammatory cytokine IL-12 [30].

Fimbriae (FIM) are polymeric protein structures located on the surface of the bacterial cell and are also part of the antigens that make up the pertussis vaccine.

Pertactin (PRN) is a highly immunogenic, surface-associated protein that is exported to the outer membrane, where it undergoes proteolytic cleavage. PRN participates in attachment to facilitate eukaryotic cell binding and invasion, and is recognized as one of the nonfimbrial agglutinogens. Antibodies to it are found after natural disease and vaccines containing this protein.

Studies from The Netherlands showed that genetic variations in PRN (and PT) molecules exist, with a shift over time in the circulating strains toward variants not represented in the pertussis vaccines used in the community. The PRN-deficient isolates were genetically diverse and different mutations were found to inactivate the PRN gene, suggesting that PRN deficiency was subject to positive selective pressure.

B. pertussis isolates not expressing PT or PRN were detected for the first time in 2007 in France. Non-expression was the result of deletion of the entire PTX locus, insertion of IS481 in the PRN gene, or deletion of a part of the PRN gene. These isolates were found to be less pathogenic in animal and in vitro models.

A study evaluated *B. pertussis* isolates collected from four Australian states during a pertussis outbreak from 2008 through 2010. A total of 194 organisms were typed by single-nucleotide

polymorphism analysis; multilocus variable number tandem repeats analysis; and fim3, prn, and ptxP sequence analyses. Results suggested increasing selective pressure favoring alleles not contained in the acellular pertussis vaccines used in Australia. Another report from Australia found that 96 (30%) of 320 *B. pertussis* isolates collected from 2008 to 2012 did not express PRN. Multiple mechanisms of PRN inactivation were documented, including IS481 and IS1002 disruptions, a variation within a homopolymeric tract, and deletion of the PRN gene [33-35]. These findings suggest that PRN-deficient *B. pertussis* arose independently multiple times, rather than representing expansion of a single PRN-negative clone. This pattern is consistent with continuing evolution of *B. pertussis* in response to vaccine selection pressure.

Reports from the United States showed that the prevalence of PRN-deficient *B. pertussis* has continued to increase since 2012. Patients infected with PRN-producing strains were significantly younger, more likely to report apnea, and more likely to be hospitalized (6% in the PRN-producing group compared to 3% in the PRN-deficient group). Data suggest that loss of PRN may confer selective advantage, while possibly decreasing clinical severity modestly.

There has been concern that the effectiveness of PRN-containing acellular pertussis vaccines might be reduced against PRN-deficient strains. However, the most recent vaccine effectiveness data from the CDC found that the effectiveness of acellular pertussis vaccine against PRN(-) and PRN(+) strains did not significantly differ [36].

Adenylate cyclase toxin (ACT) is a calmodulin-sensitive adenyl cyclase/hemolysin. It can trigger the host's immune response but is not used for the packaging of vaccines [35]. The adenylate cyclase (AC) domain activated, once it enters the cell, by binding to calmodulin (a cytosolic protein) and that result in catalyzing the synthesis of c-AMP from ATP. This increase in c-AMP levels alters normal cellular calcium homeostasis and is involved in host cell damage. Hemolysin domain, on the other hand, create selective pores for cations that contribute to the cytotoxic action of ACT in macrophage cells, thus cooperating with c-AMP in the induction of cell death. Moreover, the activity of ACT leads to rearrangements of the macrophages' cytoskeleton, destabilizing the structure of the membrane and inhibiting the complement-mediated phagocytic action. In tracheal epithelial cells, ACT induces the synthesis of high levels of IL-6 which plays a primary role in the pathogenesis of inflammation [37,38].

Dermonecrotic toxin (DNT) is a heat labile toxin, consisting of a subunit A and a subunit B, which induces peripheral vasoconstriction with consequent ischemia and damage to the respiratory mucosa.

Lipooligosaccharide (LOS) has a pyrogenic, mitogenic and toxic action. It can also activate and induce TNF in macrophages. The different structure of the O antigen could be the reason why

immunity induced by *B. parapertussis* is protective against infections from both species, while immunity induced by *B. pertussis* protects against subsequent infections of the same species, but not from those of *B. parapertussis*. It is assumed that the O antigen could inhibit the binding of antibodies to the bacterial surface and therefore allow *B. parapertussis* to avoid the immunity induced by the previous vaccination and still cause the disease [30].

Tracheal cytotoxin (TCT) is a monomeric peptidoglycan produced by Gram-negative bacteria during the mechanisms of destruction and reconstruction of the bacterial wall during their growth. TCT causes mitochondrial swelling, destruction of tight-junctions, ciliostasis and damage to ciliated epithelial cells. It also has the property of interfering with DNA synthesis in tracheal epithelial cells, preventing their regeneration [37].

There are four phases to describe the pathogenetic mechanisms of infections sustained by pathogenic bacteria in general and by *B. pertussis* in particular: 1) adhesion, 2) evasion of host's defenses, 3) local damage, 4) systemic manifestations [30]. The virulence factors of *B. pertussis* have already been discussed.

The infection begins with the adhesion of the microorganism to the cilia of the epithelial cells of the upper respiratory tract. Several factors (FHA, FIM, PT, LOS) as previous described are involved in promoting this process. Given the presence of many factors to perform these functions, it is difficult to establish which protein plays a primary role. Indirect information comes from efficacy studies carried out on pertussis vaccines. For example, a vaccine containing the FIM2, FIM3, PT, FHA and PRN proteins is significantly more effective than a vaccine containing only the PT, FHA and PRN proteins. This study suggests that FIM may have a primary role as adhesins and that antibodies directed against these antigens can block the adhesion of *B. pertussis* to host cells [30].

The evasion of the host's defenses is facilitated by ACT and PT. In particular, ACT compromises the effectiveness of the phagocytic mechanism against the pathogen, while PT performs its action by assisting the inhibition of phagocytosis and the migration of lymphocytes and macrophages to the sites of infection.

Local damage to ciliated epithelial cells is considered the effect of TCT and DNT. Ciliostasis and local damage to tracheal cells represent the pathogenetic mechanism of paroxysmal cough typical of pertussis disease [30].

Systemic manifestation caused by *B. pertussis* are leukocytosis with lymphocytosis caused by the pertussis toxin, which does not allow lymphocytes to migrate into the upper respiratory tract and therefore remain in the circulation. Moreover, encephalopathy is described but most likely it is an event due to anoxia secondary to apnea episodes that can follow paroxysmal cough [4].

1.3. Risk factors, immunity and breastfeeding role

Circulation of *B. pertussis* persists despite the high diffusion and semi-optimal adherence to vaccination [26].

In addition, a resurgence of the disease has been observed among adults and adolescents, which are a source of infection for unvaccinated (or not fully vaccinated) children [39]. It is demonstrated that anyone is at risk of contracting pertussis, with the exclusion of course of all those who have been vaccinated or have contracted the disease in recent times, also given the data that suggest the decrease in the effectiveness of vaccination already after 5- 6 years [19].

Identifying the precise sources of contagion and determining which ones contribute most to the development of the disease in infants and children would be important to reduce morbidity and mortality in this age group and to help determine which groups could become specific targets for vaccination to decrease the spread of the disease in every group of age [1].

Pertussis-naive individuals are completely susceptible to infection (infection defined as bacterial overgrowth sufficient to cause a specific immune response), but infection does not necessarily manifest as a "typical" disease. It is estimated that about 25% of infected immune subjects manifest the disease in an atypical or asymptomatic form.

Vaccination protection would appear to persist for at least 5-6 years after immunization with acellular vaccine, but the protection induced by the infection may last longer. The drop in immunity induced by the vaccine is a gradual process, caused by the re-emergence of the infection in an asymptomatic form and by the circulation of *B. pertussis* strains not included in the vaccine [40].

The role of adolescents and adults in the transmission of the infection seems to increase with the increase in vaccination coverage of children and can reach levels > 50% among all sources of transmission. In those areas with lower vaccination coverage, the main source of infection for newborns is still maintained by children.

Among cohabiting adults, mothers are the major source of transmission for their children; other adults potentially a source of transmission are health workers, mainly those operating in pediatric care [40].

Since 2010, many studies have also taken into consideration passive immunity transmitted by the mother through placenta and breastfeeding and the role of vaccination in pregnant women in order to allow greater immunization of the newborns [41-43]. Pertussis immunisation during pregnancy has been recommended in several high-income countries in order to protect young infants from severe form of pertussis disease. In Italy, it is recommended between 27- and 36-weeks gestation. The strategy is safe and effective, based on the currently available data. There are indications of a blunting

effect by the vaccine induced maternal antibodies, on the infant immune responses to aP vaccines, yet the clinical meaning is unclear [44].

Certainly, a more direct way to protect young children could be to vaccinate them in neonatal age, although the timing of vaccination should be evaluated in order to maximize its success [43,45].

It is known that mammalians protect their progeny during the first days/months of life through the transfer of maternal antibodies. At birth, the still immature immune system of the newborn faces with thousands of different commensal and pathogenic microorganisms, which colonize its body surfaces and mucosal epithelia. In this phase, the adoptive transfer of the maternal immune experience helps the newborn to survive and generate its own immune defenses.

Before birth, the mother transfers to the fetus her systemic memory (serum IgG), which will protect the child during the first months of life, although rapidly decaying between three and six months of age. After birth, newborns can only receive the mucosal immune memory of the mother through the IgA antibodies transferred with the breast milk, which contains all the nutrients and vitamins needed for neonatal growth and activity [21].

It is globally recognized that breastfeeding is included among the protective factors for respiratory infections in infants and this protective role against respiratory infections has been lengthily demonstrated in developing countries but also in high resource countries [23,46].

Breastfeeding protection seems to be time dependent: different studies showed that infants breastfed for < 4 months had a higher risk of hospitalization for infectious diseases in the first year of life compared with those who were breastfed for more than 4 months [21-23,47]. Fewer are the studies analyzing the protective role of breastfeeding in the first 3 months of life. Contrary, Pandolfi et al, in their recent study conducted a multivariable analysis identified exclusive breastfeeding as a risk factor for respiratory tract infections [21].

Evidence on the protective role of breastfeeding against infections of the gastrointestinal tract is more robust compared to findings on the protection from respiratory infections [47]. This has an immunological explanation; breastmilk contains protective factors such as immunoglobulins, lactoferrin, and lymphocytes. Immunoglobulins ingested through breastfeeding confer a direct protection against microorganisms localized in the gastrointestinal tract, which are bound by ingested breast milk IgA. Ingested immunoglobulins should be absorbed through the intestinal mucosa and move to other areas through the bloodstream. Nevertheless, some evidence shows that the process of intestinal IgA absorption is not effective [48].

Cytokines, defensins, and lactoferrin, produced by the innate immune system of the mother, lead to a wide-range protection, while secretory IgA defend the child only from pathogens known by

the immune system of the mother. Milk IgA derives from plasma cells that migrate from mucosal sites to the mammary gland at the end of pregnancy and during lactation [49,50].

Similar to natural IgA at mucosal sites, natural IgM in the serum has not only the function of first-line defense against infection but also the function of trapping antigen into complexes rapidly delivered to follicular dendritic cells to initiate and maintain the germinal center (GC) reaction.

Vaccinations, similar to natural infections, trigger the GC reaction leading to the generation of two cell types: long-lived memory plasma cells and switched memory B cells. Memory plasma cells continuously secrete their antibodies ensuring the presence of pre-formed specific antibodies in the serum. Memory B cells rapidly react to a renewed antigen encounter with proliferation and plasma cells formation to increase the concentration of specific antibodies and prevent re-infection and disease [21,51].

1.4. Clinic and complications

Pertussis clinical presentation is affected by several factors, the most important are age, previous immunization or infection and antibiotic treatment [1,52,53].

For this reason, the classic illness, characterized by three stages (catarrhal, paroxysmal and convalescent), usually occurs as primary infection in unimmunized children and is dominated by the typical paroxysms (a series of 5–30 coughs during a single expiration), frequently followed by post-tussive vomiting and by a massive inspiratory effort which results in the classic ‘‘whoop”. Paroxysms can be associated with apnoea, cyanosis, salivation, lacrimation and distension of neck veins [1,2].

Most infants and children with classic pertussis have leukocytosis (20,000–100,000/mm^3) with an absolute lymphocytosis in peripheral blood, which is usually present from the beginning of paroxysmal phase for 3–4 weeks and is directly correlated with disease severity [54].

This is a rapidly evolving combination of secondary bacterial pneumonia, severe pulmonary hypertension, cardiopulmonary failure and neurologic involvement that is more common in infants younger than 1 year old and in 80% of cases leads to death [4,55-57]. Other complications could affect neurological system such as seizures and encephalopathy likely secondary to hypoxia, cerebral bleeding or secondary infections. Paroxysmal cough episode could lead to a sudden increase in intrathoracic and intraabdominal pressure causing pneumothorax, pneumomediastinum, subcutaneous emphysema [4,54-58].

Clinical presentation of pertussis in previously immunized children or infected adolescents and adults is atypical and often asymptomatic, with the main symptom being persistent cough. These categories serve as a reservoir for infection of infants and children. Neonates and infants less than 6

months of age can develop an atypical illness too: cough and whooping may be absent and the clinical picture is dominated by recurrent episodes of apnoea, cyanosis and bradycardia [10].

Pertussis can be especially difficult to diagnose in children under 1 year of age during the winter season, when other pathogens, such as the respiratory syncytial virus (RSV) and other respiratory viruses circulate: in these difficult cases, pertussis acute respiratory symptoms can overlap with those of bronchiolitis [59,61]. Furthermore, there are conflicting data on the prevalence of *B. pertussis* in infants presenting with bronchiolitis, ranging from 0.6% to 20% [20]. Complicating more the differential diagnosis is the co-infection with respiratory viruses frequently detected in infants with *B. pertussis*, with percentages of co-infection ranging from 0.2% to 23% [62-64]. However, clinical features of cases with co-infections, often, do not differ from those with only *B. pertussis* infection [65]. Although ample evidence confirms coinfections between *B. pertussis* and other pathogens, especially viruses, the role of coinfections remains debated [66]. Most mixed infections probably arise accidentally and whether they cause more severe disease than *B. pertussis* alone remains unclear [59,60,64,67-70].

Recently, Tozzi et al, developed a data driven algorithm based on simple clinical information which reliably supports the early differential diagnosis between pertussis and other respiratory conditions in infants younger than 12 months of age [53]. This algorithmic approach might be important in clinical practice to decide the most appropriate case management in low resource settings or when laboratory confirmation is not available.

1.5. Diagnosis

According to the Centers for Disease Control and Prevention (CDC), if a suspected case of pertussis matches the clinical case definition, an epidemiologic link or specific laboratory testing should confirm it [71]. Clinical case definitions of pertussis require the presence of one or more typical clinical symptoms (paroxysmal cough for at least 2 weeks, inspiratory whoop, post tussive emesis, apnea and/or cyanosis). The specificity of case definitions is negatively influenced by the time between infection and diagnosis, previous vaccination/infection, and by increasing age of patients [54,72,73].

Microbiological diagnosis of pertussis is challenging, but the greatest sensitivity for pertussis diagnosis is obtained when combining culture, Polymerase Chain Reaction (PCR) and serologic testing [6].

Culture is the gold standard for diagnosis, but is only 20–80% sensitive and requires special media and 3 to 7 days incubation. The recovery of *B. pertussis* from nasopharyngeal swabs in culture

is a difficult procedure and is not a timely option for confirming the diagnosis rather than molecular tests that provide a higher sensitivity and quickness than culture techniques.

Real-time multiplex PCR from nasopharyngeal swabs has greatly helped in the laboratory confirmation of pertussis; when pertussis is diagnosed quickly, antibiotic treatment can mitigate symptoms and prevent transmission [4, 16, 74-76].

Direct Fluorescent-Antibody Assay

Direct fluorescent-antibody assay (DFA) of nasopharyngeal samples is a simple and rapid method that relies on microscopic visualization of fluorescent antibodies directed toward *B. pertussis* cells. Sensitivity and specificity are low, in fact DFA diagnosis should always be supported by culture, PCR, or serology [77].

Culture

Despite its low sensitivity compared to that of PCR, culture is the gold standard for pertussis diagnosis. Both for culturing and for PCR, samples taken from the nasopharynx are optimal, and these can be obtained by aspiration or by swabs [78]. Swabs should have a thin flexible shaft to be able to reach the posterior nasopharyngeal area. Oral fluid was used to diagnose pertussis by PCR in a limited study, and it was found to be as sensitive as nasopharyngeal swabs. Sampling of oral fluid is less stressful for the patient than sampling of the nasopharynx, but it is unsuitable for culture due to the high level of contamination with resident microbiota.

Bordet-Gengou and Regan-Lowe agars are the media for culture of clinical specimens to detect *B. pertussis*. Addition of the antibiotic cephalexin has been recommended to inhibit growth of contaminating bacteria. Most critical for optimal sensitivity of culture is rapid specimen transport (< 24 h) in a suitable transport medium. Growth of bordetellae is reached by incubation of agar plates at 35-37°C in a high-humidity, low CO_2 environment. Twelve-days or more incubation time is recommended for optimal sensitivity, as growth of *B. pertussis* and *B. holmesii* may be retarded. Growth should be checked daily to prevent overgrowth by contaminating microorganisms. After growth, bordetellae can be identified by biochemical reactions, or, preferably, PCR. Different bordetellae can be distinguished biochemically by oxidase, urease, and citrate utilization and microbiologically by growth rate or motility. The routine use of culture for diagnosis of pertussis has declined since the introduction of PCR methods [77].

PCR Assays

PCR assays have become an established method for detection and identification of causative agents of pertussis. Conventional PCR makes use of 2 primers that generate DNA fragments (amplicons) to allow their visualization on agarose gels. Visualization of amplicons is accomplished by capillary (or agarose gel) electrophoresis, which requires staining of DNA with an intercalating

agent (ethidium bromide). Conventional PCRs, as opposed to real-time PCRs, have the disadvantage that they are prone to contamination due to the required post-PCR analysis. For this reason, conventional PCR assays have generally been replaced by real-time PCR methods. Real-time PCR amplicons are usually chosen to be short (200 bp), often allowing only space for the forward and reverse primers and the internal probe. Short real-time products are more efficiently amplified and allow shorter elongation times, resulting in faster results [77].

For PCR, the same swab as that used for culture can be used and if only PCR is performed, swabs can be sent dry. Liquid transport medium should be avoided because of the potential for contamination of the liquid medium during transit. Swabs can be suspended in physiological saline to release DNA [77].

Insertion sequence (IS) elements are mobile DNA fragments of approximately 1,300 bp that have terminal inverted repeats and contain an open reading frame encoding a transposase (tnpA). IS elements are generally present in multiple copies in genomes, presenting excellent targets for highly sensitive PCR detection. Although IS elements specific for different Bordetella species have been found, it should be noted that, by their very nature, IS elements may be transferred between different species [77].

IS481 and IS1001 were assumed to be specific for *B. pertussis* and *B. parapertussis*, respectively, in which they occur at copy numbers of 253 and 22, respectively. These IS elements are the most used targets for detection of *B. pertussis* and *B. parapertussis*, respectively, by PCR [77].

Given that both IS481 and IS1001 have been found in other Bordetella species, the ptxP promoter region was utilized in many studies, to increase specificity and to better discriminate from *B. pertussis*, *B. parapertussis*, and *B. bronchiseptica*, but it showed a lower sensitivity than IS sequences [79,80].

To identify *B. pertussis*, and to overcome nonspecific cross-reactions with other Bordetellae, the combination of IS481 and the promoter region for pertussis toxin, ptxP, has been used most often [81-83], but also IS1002 can be used. However, ptxP PCR suffers from a disadvantage, because due to sequence variation over the ptxP region, false-negative results may arise. The purpose of dual-target PCR is to discriminate *B. pertussis* from other Bordetella species and thus to increase the specificity and sensitivity. For optimal sensitivity of multitarget PCRs, IS1002 may be used in addition to IS481 and IS1001. In this case, positive results for both IS1002 and IS481 are indicative of *B. pertussis*, while a positive result only for IS481 is indicative of B. *holmesii*. Furthermore, if both IS1001 and IS1002 PCRs are positive, this is indicative of *B. parapertussis*, while either a positive IS1001 or IS1002 PCR is indicative of a *B. bronchiseptica* infection.

The extremely high copy number of IS481 facilitates a high sensitivity of *B. pertussis* detection but, at low DNA concentrations, leads to a proportion of *B. pertussis* IS481 PCR positive results which cannot be confirmed by an additional PCR with another target present at a lower copy number [77].

Serodiagnosis of Pertussis

Serodiagnosis is one of the oldest techniques used to confirm the clinical diagnosis of pertussis. By using purified antigens, in particular Ptx, serodiagnosis has become the most sensitive way to establish infections by *B. pertussis* of sufficient duration to have mounted an immune response.

The immune response to *B. pertussis* infection starts 1 to 4 weeks after the onset of symptoms, reaching peak levels at 4 to 7 weeks. Enzyme-linked immunosorbent assay (ELISA) method permit to differentiate IgM, IgA, and IgG antibodies, with a high accuracy of quantitative measurements [4].

In studies in which IgM, IgA, and IgG antibodies were measured in sera from patients with pertussis (and various ages and vaccination histories), the common finding was that the IgG parameters were most sensitive [77].

The prevalence of *B. pertussis*-IgA antibody in the population tends to increase with age. In particular, IgA-Ptx was shown to be less sensitive than IgA-FHA. Despite the shortcomings of IgA levels for the diagnosis of pertussis, interest in measurement of IgA antibodies for serodiagnosis remains, because primary vaccinations with wP or aP in the first year of life induce IgM and IgG antibodies but do not induce IgA antibodies. Booster doses wit aP at 4-9 years in some cases induced low levels of IgA antibodies to antigens contained in the vaccine, more in wP-primed children than in aP-primed children. Nevertheless, booster vaccination of adolescents and adults with aP, has been shown to induce IgG as well as IgA antibodies, but the latter was less frequent and less strong. The measure of IgG antibodies to *B. pertussis* antigens gives the best results in terms of sensitivity for all age groups, while IgA measure may be useful to distinguish between recent vaccination and recent infection [77,84].

It is a concern the lack of an established minimum protective antibodies level; some authors described IgG anti-PT levels >5 IU/mL as potentially protective. In populations not recently vaccinated for pertussis the cut-off value <40 IU/mL) is considered negative (no IgG antibodies to pertussis toxin (PT) detected); borderline value (40-<100 IU/mL) was used to estimate the proportion of subjects infected with a likely *B. pertussis* within the last two years, whereas a value ≥ 100 IU/ml is suggestive for recent infection within the last year or recent vaccination against B. pertussis [85-88].

ELISA Oral Fluid Testing for Pertussis

The Health Protection Agency (HPA; which became Public Health England on April 1, 2013) Respiratory and Systemic Infection Laboratory (which became the Respiratory and Vaccine Preventable Reference Unit on April 1, 2013) developed an ELISA to detect IgG against pertussis toxin in oral fluid. This is an additional surveillance tool that offers higher acceptability and lower cost than other available methods; it is performed by a straightforward and noninvasive sample collection.

This test was intended to act as a surrogate for the serum antibody assay. This test detects seropositivity with a sensitivity of 79.7% (95% CI 68.3%–88.4%) and a specificity of 96.6% (95% CI 91.5%–99.1%) (16). Thus, oral fluid titers of >70 arbitrary units have a positive predictive value of 76.2%–93.2% for pertussis among children with chronic cough when used as a surrogate for the serum ELISA [89].

1.6. Therapy, prophylaxis and prevention

Pertussis standard treatment consists of the use of antibiotics such as erythromycin, clarithromycin or azithromycin. These three macrolides have equal efficacy and eradicate *B. pertussis* in 97% of cases after 2–3 days and in 100% after 14–21 days and patients are no longer infectious after 5 days of treatment [1]. Today, the preferred treatment is azithromycin (because of the shorter treatment); erythromycin use is limited in neonates because it resulted in the possibility of hypertrophic pyloric stenosis, as rare complication [90]. Both azithromycin and clarithromycin are effective. However, antibiotics are very effective in eradicating the infection, but do not improve the symptoms: starting the treatment during the catarrhal phase shortens the duration of symptoms and reduces the severity of the illness, but diagnosis is rare in this stage and in most of the cases treatment is started only after the onset of the paroxysms, when it does not impact the clinical course of the disease [72]. Corticosteroids have not shown a definite benefit in reducing severity and course of the disease, but are used in critically-ill patients. Beta2-agonists, pertussis immune globulin, cough suppressant, and antihistamines are not recommended. Supportive care includes adequate hydration and nutrition and avoid factors that provoke cough [4].

Macrolide-resistant strains of *B. pertussis* have been described; trimethoprim-sulfamethoxazole is the recommended treatment in resistant cases and patients who cannot tolerate macrolides [90].

More severe cases that present with bradycardia, apnea, respiratory failure or shock could require hospitalization in intensive care unit.

To date, the main preventive strategy for pertussis is vaccination. In Italy, vaccination scheme starts with a dose at 3 months of age, followed by other two booster doses at 6 and 11 months [9, 91].

The first vaccine was the whole cell pertussis (wP), containing inactivated *B. pertussis* organisms and having an efficacy ranged from 46% to 92% which correlated with the number of doses. Although it has a high efficacy, concerns about its adverse reactions, such as local redness (37.4%) and swelling (40.7%), pain at the injection site (50.9%), fever (46.5%), persistent crying (<1%) and febrile convulsions (<1%), led to the development of the acellular pertussis (aP) vaccines, containing one or more purified antigens and having almost the same efficacy with a superior adverse effect profile [6, 9, 92].

In the last years there is increasing evidence that neither natural infection nor vaccination give life-long immunity: several studies documented that a second episode of pertussis can occur some years after the first one. Current estimates of the duration of protection due to natural infection range from 7 to 10 years to 20 years, but there is evidence that it can be as short as 3.5 years. However, duration of protection due to natural *B. pertussis* infection can vary from subject to subject.

A similar or slightly reduced duration of protection has been calculated after immunization with wP. Different authors reported that protection due to immunization with wP was still effective in 85% of children 4 years after immunization, but was reduced to 50% in the following 3 years.

Conversely, the duration of immunity after aP immunization appears to be shorter, independent of the schedule used, the numbers, and concentrations of antigens included in each vaccine and the methods used to prepare the vaccines. Reports suggested that pertussis occurred significantly earlier in subjects fully vaccinated with aP than in those given wP; children who were fully immunized during infancy with an aP had pertussis more often in the first 4 years of life, while those given a wP were at higher risk later, mainly during adolescence.

Studies that have compared immune responses after natural *B. pertussis* infection and the administration of both wP and aP have shown that the immune stimulation evoked by aP is different from that due to natural infection and wP. Natural infection evokes both mucosal and systemic immune responses, while aP induce only a systemic immune response.

It is clear that mucosal immunity plays an essential role to prevent colonization and transmission of *B. pertussis*; consequently, aP vaccination that do not induce a valid mucosal response can prevent disease but cannot avoid infection and transmission. Animal studies have shown that natural infection is associated with a secretory IgA response in both the upper and lower airways and induction of resident memory T cells. Moreover, it has been reported that IL-17 and IFN-g-secreting CD69+CD4+ resident memory T cells were expanded in the respiratory tract after *B. pertussis* challenge of mice immunized with wP, but not aP vaccines [93-95].

However, natural infection was associated with the most persistent protection against nasal colonization and this correlated with potent induction of nasal tissue resident memory T cells. These

animal data suggest that the lack of mucosal immune response after aP administration might explain its lower efficacy when compared to wP and the shorter duration of protection compared to both wP vaccination and natural infection. In particular, natural infection and wP induce antibodies of the IgG1, IgG2, and IgG3 subclasses, with marginal production of IgG4, suggesting a strong Th1 response; conversely, the immune response after aP evoke a mixed Th2 and Th17 response, with a production of IgG1 and IgG4 antibodies, and high concentrations of IL-4 and IL-5 and low amounts of IFNγ [93].

Protection given by aP-primary immunisation wanes after 2-3 years and the risk of pertussis infection increases by 33% every year since the last dose [94]. Furthermore, protection after boosters is shorter in individuals who received aP instead of wP-primary vaccination, explaining the increased incidence of pertussis among adolescent and adults [9]. Thus, it is plausible that asymptomatic transmission of *B. pertussis*, together with the shorter duration of protection in individuals immunised with aP vaccines, may drive pertussis outbreaks [96]. For these reason, new vaccination strategies have been implemented across different age groups to protect infants in the first months of life, when the disease is life-threatening [13,97].

Booster doses in adolescents and adults

The waning immunity following aP vaccination leaves adolescents and adults susceptible to *B. pertussis* infection, making them a source of transmission to young infants. For this reason, since 2006, the CDC recommended routine vaccination with tetanus toxoid, a reduced amount of diphtheria toxoid and aP (Tdap) for adolescents. From that time, pertussis incidence in adolescents declined, but no relevant impact on infant disease has been found. This may be caused by the insufficient vaccine coverage, or due to the incapacity of Tdap to prevent asymptomatic infection and transmission of *B. pertussis*. For these reasons, decennial boosting with Tdap is now recommended in several countries included Italy [98,99].

Vaccination of pregnant women

It was demonstrated low levels of pertussis specific antibodies in neonates; they showed that, although pertussis antibodies efficiently pass the placenta, maternal levels are too low and rapidly decline in the infant serum, leaving newborns without protection. Studies on the baboon model have shown that maternal vaccination with aP vaccine is associated with efficient transplacental transfer of IgG to the newborn and protects the infant from *B. pertussis* already at 5 weeks of age [76, 100].

Further studies on humans have shown that maternal vaccination is associated with significantly higher levels of pertussis antibodies at birth in the mothers and infants. The protection probably arises from the direct effect of the antibody transfer by the placenta and the indirect effect of protecting the mother from the infection, preventing infection transmission to infants. It was,

however, demonstrated that maternal antibodies against pertussis decrease after one year and, for this reason, it is recommended to repeat Tdap vaccination with subsequent pregnancies regardless the previous history of pertussis immunization, optimally between 27- and 36-week gestation [41, 101]. Furthermore, Eberhardt et al. showed that early vaccination (13-25th gestational weeks) significantly increased anti-pertussis antibody titers in the newborn, allowing even preterm infants to benefit from maternal immunizations [102]. Finally, higher levels of anti-PT IgA have been found in the breast milk of vaccinated comparing with non-vaccinated mothers [103].

The ‘‘cocooning” strategy

Cocooning is a strategy designed to reduce the risk of infection in infants too young to be vaccinated through the immunization of their household contacts [9]. Many studies suggest that parents are the source of infection in 50–55% of cases, siblings up to 20% and grandparents almost 10% [104]. The cocooning strategy has been recommended in the US since 2006 and later in several other countries.

Nevertheless, aP vaccine did not showed to prevent colonization of *B. pertussis* and its transmission to infant; furthermore, the adherence to this strategy is limited, because parents have insufficient knowledge about pertussis and, finally, the source of infection is unknown in up to 50% of cases. Due to these limitations and to the high cost of this strategy, evidence suggests preferring maternal immunization to cocooning in terms of cost-effectiveness [105].

Neonatal vaccination

Different studies have shown that neonatal immunization with DTaP vaccines results in elevated anti-*B. pertussis* antibody titers, demonstrating that neonates are able to mount immune responses to aP vaccines [100]. However, vaccination with DTaP at birth is associated with significant increased adverse events following booster doses of DTaP, whereas this association has not been demonstrated after monovalent aP administration [106]. To date, an alone aP vaccine is not available and data on neonatal vaccination safety are not sufficient to recommend this strategy, thus further investigations are required [9]

Development of new pertussis vaccines

Until now described current aP vaccines are not controlling pertussis as well as desired. For this reason, different solution to develop new vaccines have been proposed [107, 108].

– Vaccines containing only aP antigens: removing diphtheria and tetanus toxoid components would permit more frequent pertussis booster doses and neonatal vaccination

– Changes in the vaccine antigens, for example modifying their content and increasing PT dose, or replacing them with the antigens of the new circulating *B. pertussis* strains

– Adding new antigens (ACT, etc.) to extend the antigenic coverage and the duration of protective immunity

– Implementing different delivery system to stimulate different types of immune response, especially mucosal immunity.

2. RATIONALE AND OBJECTIVES

Recent outbreaks of pertussis in industrialized countries where pertussis vaccination coverage should be high, pointed out that current prevention strategies are inadequate and the adherence not complete, in order to control the spread of disease. For this reason, pertussis is still an important public health issue.

Pertussis primarily affects children less than 6 months of age with severe clinical symptoms especially in newborns, who pay the highest price in terms of morbidity and mortality, causing an excess of admissions to intensive care units.

In children, adolescents and adults, pertussis is frequently underestimated because it may have a mild course often with an atypical clinical picture with prolonged cough, but it is often cause of missing days of school and work. These groups of individuals are described as reservoir of *B. pertussis*, causing clusters in newborns and infants.

Pertussis disease re-emergence might be due to many factors, first of all the waning of vaccine immunity consequent to introduction of the acellular (aP) vaccine and the circulation of variants *B. pertussis* strains (different from the vaccine strains). Although it has been demonstrated that breast milk contains antibodies against *B. pertussis* few data exist on the actual protective activity of breast milk against pertussis from an epidemiologic perspective.

In this project we collected evidence to support several integrated prevention strategies.

Our 3 projects and objectives were the following:

1. Microbiological surveillance of pertussis and of analysis of variants of *B. pertussis*: to estimate the proportion of infants hospitalized with suspected pertussis, and to describe circulating variants of *B. pertussis* in order to detect antigenic differences that might not be consistent with existing strains in vaccines.

2. Protective role of breastfeeding and maternal immunity in whooping cough: to assess the protective effect of breastfeeding in correlation to the levels of secretory IgA in breast milk. In addition, we collected information about the concentration of anti-pertussis IgG, IgA and IgM antibodies and specific lymphocytes in the blood of mothers of children affected by pertussis, thus exploring the relationship between circulating antibodies in the mother and the concentration of IgA in breast milk.

3. Serum-epidemiology of pertussis in parents of patients affected by whooping cough: to study the immunological profile of families of infants with pertussis, in order to clarify the role of adults in maintaining circulation of the infection.

3. METHODS

Patients – From April 2013 and May 2015, we conducted a longitudinal case-controls double-center study, and enrolled 141 consecutive infants, hospitalized at the Pediatric Departments "Sapienza" University and at the Bambino Gesù Children's Hospital (Rome), and 235 healthy controls (HC) admitted as outpatients for hip ultrasound screening at the Bambino Gesù Children's Hospital.

Inclusion criteria of the 141 infants enrolled: age lower than 180 days, with at least one symptom among cough lasting more than 5 days, paroxysmal cough, apnea or cyanosis and post-cough vomit. Exclusion criteria: chronic diseases and genetic syndromes.

At admission all 141 infants underwent a nasopharyngeal washing obtained instilling 3 ml of sterile saline into each nostril and collected with a syringe. All samples were delivered to the Department of Infectious, Parasitic & Immune-mediated Diseases at the Istituto Superiore di Sanità (Rome) for *B. pertussis* detection.

Patients tested positive for *B. pertussis* were enrolled as cases (n=73, Pertussis), those tested negative for pertussis were enrolled as control group (n=68, Lower Respiratory Tract Infections, LRTI). No other Bordetellae as *B. parapertussis*, *B. bronchiseptica* and *B. holmesii* were detected.

Fifty-six out of the 73 positive *B. pertussis* samples were molecularly characterized, and the entire pertactin gene sequencing were performed on the 18 pertussis viable samples isolates in culture.

A total of 157 mothers from cases and controls infants, who were breastfeeding, were included in the immunological study and requested to undergo a blood sample collection and to express a sample of breast milk. Milk was obtained from 53 pertussis, 21 LRTI, and 61 HC mothers. Blood samples were collected from 57 mothers of pertussis cases, 36 mothers of infants with LRTI, and 61 HC mothers. None of the mothers enrolled in the study had been vaccinated against pertussis or recalled to have had the disease in infancy or in the last years.

A total of 167 parents from 145 infants were enrolled in the study about the serum-epidemiology of pertussis: 53 mothers and 21 fathers of the 73 pertussis cases (in 19 cases both the mother and the father of the same infant were enrolled), 32 mothers and 5 fathers of the 33 LRTI cases, and all the mothers of the 57 HC infants.

The flow chart of the patients and parents enrolled in the study is shown in Figure 2.

Demographic and clinical data – All parents of cases and controls, at enrollment, were asked to answer to a structured questionnaire seeking the following demographic data: age at admission (in months and days), gender, ethnicity, gestational age, birth weight, type of delivery, kind of feeding at symptom onset (exclusive breastfeeding, partial breastfeeding and artificial feeding), number of

households, presence of siblings, level of education and employment for each parent, presence of smoking parents, patient's immunization status against pertussis.

For pertussis and LRTI infants, we also recorded the following clinical variables at admission in the hospital: use of macrolides or steroids before admission, days of cough before the admission and after the discharge, days of hospitalization, presence of fever (body temperature > 37.5°C), and clinical presentation (cough, episodes of paroxysmal cough, cyanosis, apnea, post-cough vomit).

In order to take into account possible sources of infection in infants with pertussis, the parents of the pertussis group were asked to report the presence of a family member (parent, sibling, grandparents, uncle/aunt) with cough lasting at least 3 weeks before the admission of infants.

Before enrollment, all children's parents gave written informed consent to participate in the study, which was approved by institutional review boards of both hospitals (Policlinico Umberto I: protocol 213/14, 3085/13.02.2014; Bambino Gesù Children's Hospital: protocol n. RF-2010-2317709), and the study was performed following the guidelines of the Declaration of Helsinki.

All data were recorded in an electronic database.

Detection of Bordetella pertussis by RT-PCR, culture and genes sequencing - *B. pertussis* DNA was extracted with QIAamp DNA minikit (QiaGEM, Hilden, Germany) and amplified with the "Bordetella Real-Time PCR" kit (Diagenode Diagnostics, Liège, Belgium) which target is IS481.

To prevent misdiagnosis of *B. holmesii* as *B. pertussis*, all samples positive for *B. pertussis* were confirmed with a specific Real Time PCR assay for *B. pertussis* using the ptxP (promoter of pertussis toxin gene) as target [109]. All Real Time PCR assay was performed using the LightCycler 2.0 (Roche Diagnostic) and data were analyzed with LightCycler software (version 4.0, Roche Diagnostic).

Positive samples for *B. pertussis* were cultured on charcoal agar plates (Oxoid England) containing defibrinated sheep blood at 10% and incubated at 35 °C up to 7 days and inspected daily, as previously described by our group [60]. For each sample, selective and non-selective medium containing cephalexin (40 mg/l) were used. Gram-staining determination and oxidase production assay were performed. The identification was confirmed using specific anti-*B. pertussis* agglutinating antiserum (Murex Diagnostics, France). Sequence based typing was performed on DNAs extracted from bacterial isolates, using the QIAamp DNA minikit. The genes of pertactin (prn), pertussis toxin promoter (ptxP) and pertussis toxin subunit A (ptxA) were sequenced using methods described by Mooi and Schouls et al [110, 111]. Full length pertactin gene sequencing was performed on viable isolates using primers and protocol as described by Barkoff et al [112]. PCR amplifications were carried out using Mastercycler personal thermal cycler (Eppendorff, Hamburg, Germany). All

amplification products were analyzed by electrophoresis and purified with QIAquick purification columns kit (QIAGEN) for subsequent sequence analysis by Sanger method. Sequences were analyzed with BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) or MUSCLE (http://www.ebi.ac.uk/Tools/msa/muscle/).

Multilocus variable number tandem repeat (VNTR) analysis (MLVA) is a useful technique for outbreak surveillance. It is a method employed for the genetic analysis of bacterial microorganisms, and contributes to forensic microbiology through which the source of a particular strain might eventually be traced back. In a typical MLVA, a number of well-selected and characterised (in terms of mutation rate and diversity) loci are amplified by RT-PCR, so that the size of each locus can be measured, usually by electrophoresis of the amplification products together with reference DNA fragments (a so-called DNA size marker) [113]. MLVA analysis was carried out on 18 *B. pertussis* isolates. To determine the repeat count for each locus, the sequence of 6 loci was performed as described previously by Schouls et al [111,112,114]. The assignment of MLVA type was based on the combination of repeat counts for VNTRs 1, 3a, 3b, 4, 5, and 6 and was consistent with international nomenclature (http://www.mlva.net/).

Detection and measure of IgA against B. pertussis antigens in breast milk - Whole-milk aliquots were stored at −20°C in a frost-free freezer until assayed for pertussis-specific antibodies. An in-house assay was developed to measure breast milk IgA. *B. pertussis* antigens, purified PT (NIBSC, Potters Bar, UK), filamentous hemagglutinin (FHA) (NIBSC, Potters Bar, UK) and pertactin (PRN) (List Biological Labs, Campbell, CA) were used for coating (5 µg/mL). As control, we also measured milk IgA specific for pneumococcal polysaccharides using precoated plates (Binding Site, Birmingham, UK). Human anti-IgA-horseradish peroxidase (HRP) was used for detection, and optical density (OD) was measured at 2 different time points. Breast milk was used at 1:10 and 1:30 dilutions and tested in triplicates.

Binding of milk-IgA to bacteria by fluorescence-activated cell sorting flow cytometry –The method is represented in Figure 3. The study included 8 frozen barcoded bacterial isolates from routine specimens (*Klebsiella pneumoniae, Klebsiella oxytoca, Pseudomonas aeruginosa, Streptococcus pneumoniae, Streptococcus salivarius, Staphylococcus aureus, Enterococcus faecalis* and *Escherichia coli*), that can be found in the microbial communities colonizing airways, gut, and skin. Bacterial isolates were cultured on Columbia agar +5% sheep blood for 24 hours at 37°C, morphologically characterized and subsequently identified with matrix-assisted laser desorption ionization time-of-flight mass spectrometry. *B. pertussis* isolated from nasopharyngeal aspirates was stored at −80°C using a system of small glass spheres in a test tube from freezing Cryobank (Mast

Diagnostics GmbH, Reinfeld, Germany). To obtain viable bacteria, a sphere with adherent bacteria was removed from the frozen test tube using a sterile needle and immediately placed on Bordetella Selective Agar (Biolife Italian Srl, Milan, Italy) where the bead was streaked on the surface of the soil to distribute the bacterial cells and incubated at 35°C in a humid atmosphere for approximately 4–5 days. Bacterial colonies were collected using a sterile swab and mixed in a test tube with a sterile saline solution of NaCl 0.45% (Bio Merieux S.A., Marcy l'Etoile, France), to obtain a suspension of 3 McFarland.

Bacterial single colonies and *B. pertussis* suspension were diluted in 1.5 mL of phosphate-buffered saline. For each staining, 45 mL of bacteria suspension was incubated with either FACS buffer (negative control) or maternal milk (5 µL l of 1:2, 1:4 dilution in FACS buffer) and left for 20 minutes on ice. Samples were washed 3 times with ice-cold FACS buffer and then antihuman IgAfitc labeled was added for 20 minutes. After 3 washing steps, bacteria were diluted in 300 mL ice-cold FACS buffer, and data were acquired on a FACS Canto II (BD Biosciences, San Jose, CA) using forward scatter and side scatter parameters in logarithmic mode. Data were analyzed using DIVA 6.0 software (Beckton Dickinson, Franklin Lakes, NJ).

The binding of human IgA to bacteria (bacterial binding) was measured by comparing the staining for IgA of each bacterial species incubated either with FACS buffer or with milk. The frequency of bacterial binding is a semiquantitative measure of the concentration of specific antibody in the analyzed sample.

Phenotypic analysis of maternal B cells by flow cytometry

Heparinized peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll Paque™ Plus (Amersham Pharmacia Biotech) density-gradient centrifugation, counted, and stained with the appropriate combination of fluorescent labeled with anti-CD19, anti-CD27, anti-CD24, and anti-IgM antibodies and analyzed by flow cytometry. We calculated, in the lymphocyte gate, the frequency of total B cells (CD19+) and memory B cells (CD19+CD24+CD27+); we also measured the frequency of IgM (CD19+CD24+CD27+Ig+) and switched (CD19+CD24+CD27+ IgM−) memory B cells in the memory B cells gate. For each staining, at least 50,000 events in the lymphocyte gate were collected on a FACS Canto II. Data were analyzed using the DIVA 6.0 software (BD Bioscience).

Maternal B cells oligonucleotides stimulation and ELISPOT

PBMCs were cultured in complete medium at a concentration of 2.5×10^6 cells/mL. A total of 0.35 µM of Class B CpG oligonucleotides (CpG B ODN2006, Hycult Biotech) (a TLR9 ligand) was added to induce the proliferation of memory B cells and their differentiation into antibody-producing cells, for 5 days. Ninety-six-well plates (MultiScreen-HA, Milipore) were coated overnight

with AffiniPure F(ab')2 Fragment Goat anti- human IgA+IgG+IgM (H + L; Jackson Immuno Research Laboratories) for the measurement of total memory B cells.

For the detection of specific memory B cells, plates were coated with the following *B. pertussis* antigens (5 µg/mL): purified pertussis toxin (PT, NIBSC, Potters Bar, UK), filamentous hemagglutinin (FHA, NIBSC, Potters Bar, UK), and pertactin (PRN, List Biological Labs, Campbell, CA). After washing with sterile PBS/0.05% Tween 20, plates were blocked for 1 h at 37°C with PBS/gelatin 1%. PBMCs, stimulated for 5 days, as described before, were collected, counted, and seeded in the pre-coated plates. Plates were left at 37°C, 2% CO2 for 4–6 h to allow antibody secretion. A total of three 1:2 serial dilutions were done starting in the first well with 5×10^4 cells for detection of total IgM, IgG, and IgA. A total of 2×10^5 cells were seeded in the first dilution well (three 1:2 serial dilutions) for the detection of B cells secreting specific antibodies. After incubation, plates were washed with dH_2O/0.05% Tween 20 (once) and PBS/0.05% Tween 20 (two times) and incubated overnight with either anti-IgM HRPO (1:1,000), anti-IgG HRPO (1:2,000), or anti-IgA (1:2,000; Jackson Immuno Research Laboratories) diluted in PBS + gelatin (1 + 0.05%) Tween 20 (Sigma). After washing twice as before, TMB substrate (ready to use from Mabtech) was used according to the manufacturer's instructions. Plates were left at room temperature to allow the blue color to develop and the reaction was stopped with dH_2O. Plates were left to dry before counting with an ELISCAN (A-EL-VIS).

***Detection of maternal and paternal IgG, IgA and IgM against pertussis* -** Blood samples were collected by capillary or venous routes using Vacutainer tubes with an integrated serum separator (Becton–Dickinson, Milan, Italy) to minimize the risk of hemolysis and to avoid blood cell contamination of the serum when transferring to standard serum tubes.

Pertussis-specific IgG against PT (IgG anti-PT) were measured in the parents' sera using the ELISA standardized within a European Sero-Epidemiology Network (ESEN) [85,114-116]. In-house reference sera and international standards (06/142 WHO International Standard Pertussis Antiserum) were calibrated against the USA-FDA standard serum (lot 3-HRP3) [115]. A total of eight twofold dilutions per sample were used in the ELISA assay to calculate the titre and results were expressed in IU/ml. The minimal level of detection was 1 IU/ml. No correlate of protection has been established for pertussis, but IgG anti-PT levels >5 IU/mL has been considered potentially protective. In populations not recently vaccinated for pertussis the cut-off value of ≥ 50 IU/ml was used to estimate the proportion of subjects infected with *B. pertussis* within the last two years, whereas a value ≥ 100 IU/ml is indicative for infection within the last year [85-88]; we utilized 100 IU/ml cut off to define parents putative responsible of pertussis infection transmission to their infants.

Pertussis toxin-specific IgA (IgA anti-PT) was measured in sera using a commercial ELISA kit (Anti-Bordetella Pertussis Toxin IgA, Euroimmun AG) according to the manufacturer's instruction. Data are expressed in IU/mL, and the lower detection limit was 0.7 IU/mL. IgA anti-PT concentration increases during *B. pertussis* infection but decays more rapidly compared to IgG. Cut-off of 20 IU/ml is indicative of a recent pertussis infection.

Pertussis toxin-specific IgM (IgM anti-PT) was measured in sera using an in-house ELISA. Briefly, Immulon® Microtiter™ 96-Well Plates (Termofisher) coated with 200 ng of purified PT antigen [114] were incubated with 100 mL/well of 1:100 prediluted sera for 2 h at 28°C. After washing, peroxidase-conjugate anti-human IgM Ab (Jackson Immuno Research Laboratories) was added to the wells and plates incubated O.N. at RT. After incubation, the plates were washed and the substrate solution added (OPD; Sigma-Aldrich). The reaction was stopped after 20 min by the addition of 10% sodium dodecyl sulfate, and plates were read at 450 nm wavelength. IgM anti-PT is not usually included in the tests for the diagnosis of pertussis; since no specific international standards for IgM anti-PT are available, optical density (OD) values are reported.

3.1. Statistical analysis

Statistical significance was analyzed with SPSS version 25.0 (SPSS Inc., Chicago, IL, USA). Data are presented as mean and standard deviation (SD), percentages for discrete variables, median and range for continuous variable. Differences among groups were compared using non-parametric Mann-Whitney test for median comparison, while differences between means were studied through Student t-test. The χ-square test or Fisher exact test was applied to analyze categorical independent variables qualitatively.

In Italy, the prevalence of exclusive breastfeeding at 3 months has been estimated to be nearly 60%. Considering this estimate, we calculated that 50 cases and 200 controls (case-to-control ratio of 1:4) were sufficient to show an OR of 0.4 for breastfed infants with a power of 80% and a 95% confidence level.

A multivariable logistic regression analysis was performed in order to study the effect of exclusive breastfeeding (exclusive vs. partial breastfeeding or artificial feeding, at symptom onset for cases or at enrollment for controls) and duration of exclusive breastfeeding on confirmed pertussis, adjusted for the following variables: age (days), sex (male vs. female), ethnicity (Caucasian vs. non Caucasian), gestational age at birth (weeks), birth weight (kg), kind of delivery (vaginal vs. cesarean), parents' employment, parents' level of education (university degree vs. lower), parents' smoking, having more than 3 households and having at least 1 brother.

Multicollinearity between the independent variables was assessed by studying the correlation matrix and examining the tolerance and the variance inflation factor.

Wilcoxon signed rank sum test was used to study, among cases, if there was difference between the opsonization of each bacterium (*S. salivaris, E. faecalis, K. pneumoniae, P. aeruginosa, E. coli, S. aureus, S. pneumoniae, K. oxytoca*) and *B. pertussis*. The correlation between antibody levels in serum and secretory IgA in breast milk was studied through the Spearman test. Pearson correlation coefficient was used to measure the linear correlation between samples.

Comparison was done by using Odds Ratios (OR) with 95% confidence intervals (95% CI); antibody values in each study group were presented as geometric means values (GMV) along with their 95% confidence intervals (95 % CI).

P-values <0.05 were considered to indicate statistical significance.

4. RESULTS

4.1. Microbiological surveillance of pertussis and analysis of variants of *B. pertussis*

Microbiological surveillance of pertussis

Among the 141 total children hospitalized following the inclusion criteria, we identified 73 (51.8%) children (n=44 males, 60.2%) positive for *B. pertussis* and 68 (48.2%) children negative for *B. pertussis* (LRTI, n=28 males, 41.1%). Among these confirmed cases, we were able to perform culture in 18 (24.6%). Median age of confirmed cases was 45 days (range 7–165); of them, 9 (12.3%) had received one dose of vaccine, and 3 (4.1%) 2 doses before the date of symptoms onset.

In Table 1 are showed demographic and clinical characteristics of the 141 infants studied. We showed that paroxysmal cough, post-cough vomiting, cyanosis, and apnea were significantly more reported among children with *B. pertussis* compared with LRTI infants ($p < 0.05$ for all the symptoms reported).

Analysis of variants of B. pertussis

In 55 out of the 73 samples tested positive for *B. pertussis*, we had sufficient genetic material to perform molecular characterization. PtxA1 was detected in all samples; 54 samples carried ptxP3 and 2 the ptxP1 allele. Moreover, the pertactin variable regions analysis revealed the presence of prn2 allele.

MLVA typing and the entire pertactin gene sequencing were performed on 18 pertussis cultured samples viable isolates. As shown in Table 2, MT27 profile was the predominant (16/18, 88.9%) and mainly combined with ptxP3-ptxA1-prn2 genetic profile. In 9/16 samples it was identified the pertactin deficient gene due to the presence of the IS481 at nucleotide position 1613 as previous described. All the samples showed ptxP3 allele. MT28 and MT60 were represented by a single isolate, associated with ptxP3-ptxA1-prn2 and ptxP1-ptxA1-prn2 profiles, respectively.

4.2) Protective role of breastfeeding and maternal immunity in whooping cough

Epidemiological role of breastfeeding

The analysis of the demographic characteristics (Table 3) showed that HC infants were older than pertussis cases (median 2.26 [0.23–4.08] vs 1.53 [0.23–3.49] months; $p < 0.001$), a higher number of HC' parents had a university degree compared with cases' parents (mothers 40.4% vs 21.3%, p= 0.006; fathers 32.3% vs 16.7%, p=0.017), household was composed by 3 or more adults in 25% of cases and in 10% of the HC group (p=0.003), and at least 1 sibling was present in 64% of cases and 44% of HC families (p=0.006). Moreover, exclusively breastfed was present in 57.4% of the pertussis cases compared to 48.5% of HC (p=0.007), and the mean duration of breastfeeding was 31.3 days (SD ± 30.11) in the pertussis group and 39.7 days (SD ± 35.26) in the HC group.

According to our multivariable analysis (Table 4), exclusive breastfeeding was not a protective (nor a risk) factor for pertussis (OR: 1.2; 95% CI: 0.31–4.67), compared with partial breastfeeding/artificial feeding, and the duration of breastfeeding was not associated with the risk of pertussis (OR: 1.01; 95% CI: 0.98–1.04). It is worth noting that infants with at least 1 sibling had a more than double risk to get pertussis (OR: 2.5; 95% CI:1.21–5.35).

IgA in breast milk

ELISA analysis (Table 5) showed that breast milk IgA against pertussis purified antigens (PT, FHA and PRN) were not significantly different between the two groups. On the contrary, IgA binding to *B. pertussis* was significantly higher in cases compared to controls (median: 4.35 vs 2.80; p=0.004). Comparing IgA in the milk of the mothers of the three studied groups (N=53 pertussis group; N=21 LRTI group; N=61 HC group), we reported that IgA binding with *B. pertussis* was present in low amounts compared to IgA against other bacterial species (Figure 4). On the contrary (Figure 5), in the milk of pertussis mothers, IgA antibodies reacting with *B. pertussis* were significantly increased compared to HC and LRTI mothers (p=0.001 and p=0.003 respectively).

Maternal serum IgG, IgA, IgM against B. pertussis antigens

A total of samples provided from mothers of 57 pertussis, 17 LRTI, and 50 HC samples could be evaluated for IgG anti-PT; whereas 20 pertussis, 15 LRTI, and 15 HC samples could be evaluated for IgA anti-PT and IgM anti-PT. In Figure 6A and 6B, we showed that IgG anti-PT and IgA anti-PT were present at significantly lower concentration in LRTI and HC mothers than in pertussis mothers ($p < 0.01$ and $p < 0.001$ respectively). In Figure 6C, is showed that in the pertussis group, mothers with higher IgG anti-PT had significantly higher level of IgA anti-PT than mothers with lower IgG anti-PT, indicating an ongoing specific immune response against *B. pertussis* infection. Finally (Figure 6D), IgM anti-PT were increased in the serum of pertussis mothers but also in the serum of LRTI mothers when we compared them to HC mothers ($p < 0.05$).

Maternal peripheral blood B-cell

The analysis of comparation of the peripheral blood B-cell of HC, LRTI, and pertussis mothers (Figure 7), showed that there was no difference in the frequency of total B cells and switched memory B cells (SW) between the three groups. Finally, in HC mothers, IgM memory B cells were significantly higher than switched memory B cells (p=0.014) (Figure 7A). No differences in the frequencies of memory B cells secreting IgM, IgA, and IgG were found among the three groups studied (Figure 7B).

To conclude immunological maternal analysis, in Figure 8, we analyzed the frequency of memory B cells secreting IgG, IgA and IgM against the three different pertussis antigens (PT, FHA, and PRN). In the group of pertussis mothers, the number of B cells producing IgG against the three

different antigens was significantly higher compared to HC mothers (PT $p < 0.0001$; FHA $p < 0.0001$; PRN $p < 0.0001$) and LRTI mothers (PT p=0.0142, FHA p=0.0143, PRN p=0.0484) (Figure 8A). Moreover, we observed a significant increase in the frequency of the B cells secreting IgA against pertussis antigens between pertussis and HC mothers (PT p=0.0005, FHA p=0.0003, PRN p=0.0011; Figure 8B). Finally, B cells secreting IgM against the three antigens (Figure 8C) were significantly higher in pertussis and LRTI mothers if compared to HC mothers (pertussis vs HC: PT and PRN $p < 0.0001$, FHA p=0.0002; LRTI vs HC: PT p=0.0027, FHA p=0.0044, PRN p=0.0304).

4.3) Serum-epidemiology of pertussis in parents of patients affected by whooping cough

Parents serology against B. pertussis antigens

The last chapter of our study focused on the measure of IgG anti-PT levels in sera from parents of the three studied groups (Figure 9). Among the 74 parents of pertussis group infants, 30 individuals (40.5%) had a level IgG anti-PT recognized positive for a recent infection (IgG anti-PT ≥ 100 IU/ml). No cases of recent pertussis infection were detected among the 37 LRTI parents, and 2 cases were detected among the 57 HC parents ($p < 0.0001$ and p=0.0002 respectively).

In order to assess the role of maternal or paternal pertussis transmission in infants, we compared IgG anti-PT levels between mothers and fathers of the 19 pertussis cases of which both parents had been enrolled (Figure 10A). The number of recently infected mothers and fathers was similar (6/19; 31.6% vs 5/19; 26.3%), with a geometric mean value slightly higher in the mothers' group (mothers 52.1, 95% CI 24.8-109.4 vs fathers 27.9, 95% CI 12.1-64.3), but not significantly.

When values of the 19 couples enrolled were compared (Figure 10B), we found that in 3/19 (15.8%) both parents had IgG anti-PT ≥ 100 IU/ml; in 4/19 cases (21.0%) the mother only had IgG anti-PT ≥ 100 IU/ml; in 3/19 cases (15.8%) the father only had IgG anti-PT ≥ 100 IU/ml; in other couples the parents had not indication of recent infection.

Seventy-three out of the 74 parents of cases answered the questionnaire to report whether they were having cough symptoms longer than 3 weeks at the moment of infant hospitalization. As shown in Figure 11, 21/73 (28.8%) reported prolonged cough; of these 14 (66.7%) had IgG anti-PT levels indicative of a recent infection. Among the 52 parents who did not report coughing symptoms, 15 (28.8%) had IgG anti-PT ≥ 100 IU/ ml (geometric mean value parents with symptoms 141.3, 95% CI 87.1–229.46 vs. parents without symptoms 26.9, 95% CI 17.6–41.2, p=0.0001).

Worth of note, 15/29 (51.7%) parents with IgG anti-PT ≥ 100 IU/ml did not report symptoms of prolonged cough at enrollment, which indicated asymptomatic or pauci-symptomatic pertussis infections in about one half of recent infected parents.

Symptoms' evaluation in relatives of infants with pertussis

As shown in Table 6, four categories of parents were identified and the combination of serology with symptoms allowed to identify a parental transmission in 31/55 pertussis infants (56.4%).

Moreover, at enrollment, 55/73 parents provided information about prolonged cough in other family members, in order to focus on the familial sources of infection. The following symptomatic relatives were reported: 11 siblings, 3 grandparents, and 3 uncles/aunts.

This information allowed to find out that in 17/24 pertussis infants whose parents were both asymptomatic and negative to serology, symptoms were reported in another member of the family, and a source of infection could be identified in 48/55 relatives (87.3%).

5. DISCUSSION

Our project leads to identify a prevalence of more than 50% of pertussis cases among a population of infants younger than 6 months enrolled. Infants with pertussis recorded more frequently respiratory symptoms if compared with infants tested negative and *B. pertussis* MT27 strain is the profile (ptxA1-ptxP3-prn2) identified in 88% of the samples cultured.

Moreover, we demonstrated that breastfeeding does not exert a protective role against pertussis infection and supported epidemiological findings with immunologic data, showing a low immunologic activity of breast milk against *B. pertussis*, compared with other pathogens. In addition, mothers of infants with pertussis have pre-existing pertussis-specific antibodies and memory B cells and react against the infection with a recall response increasing the levels of specific serum IgG and the frequency of all isotypes of memory B cells secreting Ig.

Finally, we found that 40% of parents of infants hospitalized with pertussis infection had a serological evidence of recent infection, regardless of symptoms. Moreover, 30% of parents who did not report symptoms had a serological evidence of recent infection. The combination of these results with the epidemiological survey allows us to suppose a source of familiar contagion in almost 90% of infants with pertussis.

Pertussis is a severe and even lethal disease for children too young to be vaccinated, and represents a re-emergent public health issue. In Italy, the vaccination against pertussis has been recommended since 1962 with the whole pertussis vaccine; since 1995, acellular vaccines were introduced, and since 1999 the Ministry of Health recommendations on pertussis immunization provide for a two-dose primary series at 3 and 5 months of age and a booster at 11 months; a pre-school booster dose between 5 and 6 years and a further booster between 11 and 18 years. The vaccine coverage for the primary series reached the maximum in 2008 with a 96.7 % coverage, declining thereafter and reaching a 94.9% coverage in 2019 (cohort 2017) [13,117,118]. In our descriptive

study, more than 50% of infants enrolled with respiratory symptoms were positive for pertussis, but the most of them were unimmunized.

The diagnosis of pertussis still relies on clinical symptoms, while microbiological confirmation is not performed on a large scale; this lack determines an under-recognition and under-notification of cases [10,59,107]. Our finding of a half positive pertussis infants among a population with respiratory symptoms, emphasized the need in the use of laboratory confirmation tests as criterion to confirm a clinically-suspected pertussis case. On the other hand, there is a need to simultaneously perform *B. pertussis* culture to perform genetic sequencing, that might play a role in understanding the differences among circulating pertussis isolates. Many studies have shown that variants have increased in frequency after the introduction of aP vaccine and is demonstrated that all currently circulating strains have different genotypes from vaccine strains regarding, in particular ptxA and prn genes. *B. pertussis* MT27 strain is the profile identified in 88% of our cultured samples, and was the predominant type during the past decade in Australia, Europe, USA, and Japan [110,111,119,120]. The MT27 profile expressing prn2 and ptxP3 has been extensively described worldwide, showing the potential to cause epidemics as a result of positive selection in a highly vaccinated population [112,113]. It was not surprising that the pattern ptxA1-ptxP3-prn2, has been found in our population of infants, mostly unvaccinated, and it is worth noting that other authors described that this molecular pattern was associated with severe clinical manifestations in infants [13, 121]. We missed to compare clinical differences among infants expressing different molecular patterns, in particular we found 9/16 samples with prn-deficient strains, which were found to be less pathogenic in animal, human and in vitro models, and 16/16 samples expressed ptxP3 allele, instead of the common ptxP2, were found to produce greater amounts of PT and cause more severe disease in younger infants. In our project we did not compare molecular pattern and clinical manifestations; it will be the objective of our future research [13].

Some authors described that the effectiveness of PRN-containing aP vaccines might be reduced against PRN-deficient strains. However, most recent vaccine effectiveness data from the CDC found that the effectiveness of aP vaccine against PRN(-) and PRN(+) strains did not significantly differ [36]. All these data, lead to the conclusion that more effective vaccines should include different *B. pertussis* strains.

As regards to breastfeeding, we cannot support a protective role of breast milk against pertussis infection, which showed a low immunologic activity, compared to other pathogens. No protective effect has been found for the duration of exclusive breastfeeding. Our results confirmed the findings by Pisacane et al, who analyzed data from children younger than 12 months of age, clinically diagnosed with a pertussis-like syndrome, and concluded that breastfeeding was not

protective against pertussis [122]. Compared with Pisacane's study, we focused our investigation on a highly selected population of infants, and cases were enrolled on the basis of a positive PCR on the nasopharyngeal aspirate, rather than on a clinical case definition only, but all infants enrolled in our study were younger than 4 months of age; we cannot exclude that a long duration of breastfeeding would protect children after this period of life. In fact, other authors showed a clear effect of prolonged breastfeeding (>4 months) on protection from infections but not focused on pertussis [123,124].

Along with many anti-microbial mechanisms (e.g. lactoferrin, carbohydrate components) [125], the presence of IgA, the most abundant antibody in maternal milk, is one of the mainstays of its immunologic activity. Breast milk IgA acts by binding pathogens and preventing their adhesion and penetration through mucosal membranes. It is produced by plasma cells that have migrated from the gut and the respiratory tract to the lactating breast at the end of pregnancy. Thus, the specificity of IgA in the milk has been selected by previous immune responses of the mother at mucosal sites [125]. In our study we found IgA antibodies binding bacterial species but low amounts of IgA able to bind intact *B. pertussis* or its purified components. Although binding to *B. pertussis* was significantly higher in breast milk from mothers of cases, infants were born without sufficient protection and cannot be helped to fight infection by breastfeeding. A correlation of protection for IgA in breast milk has never been studied, neither for pertussis nor for other bacterial or viral pathogens. Overall, our findings seem to suggest that there is an immunologic activity of breast milk against pertussis; nevertheless, this activity is not sufficient to exert a clinical protection against this infection. Previous studies have shown that vaccination in pregnancy may increase IgA in breast milk [126] but none of the studies published on the immunological role of breast milk against pertussis included an evaluation of epidemiological outcomes. Our study simultaneously focused on the epidemiological impact of breastfeeding and on the immunological activity of breast milk against pertussis, enhancing the value of the obtained results. Nevertheless, none of the enrolled mothers had been previously vaccinated against pertussis, as, in Italy, pertussis vaccination during pregnancy was introduced in the recommended immunization schedule only in 2018. We cannot exclude that the already demonstrated increase of IgA against pertussis in breast milk after maternal immunization might also enhance clinical protection from pertussis in the breastfed infant, but for this we will carry on further studies.

Concerning the evaluation of the mothers' immune system, we showed that mothers of the pertussis group actively reacted against the pathogen by expanding antigen-specific memory B cells and thereby increasing the production of antibodies of IgG, IgM and IgA isotype. Thus, we hypothesize that, in the adult, specific memory B cells prevent severe clinical pertussis by rapidly

producing antibodies. The detectable baseline levels of antibodies and memory B cells in control mothers (HC and LRTI) and the ability to generate a recall response might suggest that previous contacts with *B. pertussis* have occurred in the adult population and confirm that memory B cells play a major role in the adult defense. All these findings could support maternal vaccination to prevent pertussis in newborns; retrospective studies showed 91% effectiveness of DTaP vaccination of mothers for protecting newborns against pertussis in the first 2 months of life [20,127]. As the mother can exclusively transfer antibodies and no memory B cells during pregnancy, the effectiveness of maternal vaccination indicates that passively transferred IgG indeed protects against infection, but only at high concentrations. Placental transfer of anti-PT maternal antibodies is a highly efficient mechanism ensuring that specific IgG levels are higher in the newborn than in the mother [37]. Transferred antibodies rapidly decay and at 2 months of age the concentration of IgG anti-PT is decreased by 76% from the levels measured in cord blood [128].

Here, we show that IgM memory B cells secreting antibodies against PT, FHA, and PRN are significantly increased in the peripheral blood not only in the mothers of the pertussis children but also in the LRTI control group. This finding confirms that IgM memory B cells expand polyclonally as a first-line defense to every kind of infection. The increase in IgA in the milk of mothers of the pertussis and LRTI groups raises the question of whether polyclonal first-line reactions also occur at mucosal sites [51,129,130].

The last chapter of our study was directed to the identification of the source of infection in the group of cases. Our epidemiological evaluation of the parents of pertussis cases focused on the collection of serological samples; some parents refused to take the blood test. In the future we could perform to search antibodies against PT on saliva samples, a less invasive method and with good sensitivity and specificity [89].

We found that overcrowding in the household significantly increases the risk of getting pertussis. This result is in line with evidence on the role of mothers and children and, in particular, siblings, as an important source of infection for pertussis cases [105,131]. It is also well known that *B. pertussis* infects subjects of all ages; in particular, adults and adolescents may be unaware of being infected as they experience mild symptoms, undistinguished from other respiratory infections and might act as infection source for newborns and infants [117]. Awareness among physicians about this issue and pertussis circulation might lead to increased suspicion of the disease in patients with persistent coughing and, consequently, to a more frequent testing. Thus, as pointed out by Skoff et al, the cocooning strategy, recommending pertussis booster vaccination only to newborns' adult caregivers may not be suitable for effectively enhancing pertussis prevention in infants [105]; it might

reduce the disease burden of pertussis by limiting its transmission from parents [132], but is instead unable to prevent pertussis in the neonate [133,134].

In our study we showed in HC parents a 3.6% of seroprevalence of IgG anti-PT titers ≥ 100 IU/ml, in line with data from a seroprevalence study conducted among individuals in reproductive age in five Italian regions (5.1% for IgG anti-PT ≥ 100) [114]. Based on the serology, the percentage of infants with pertussis infection that had at least one parent as possible source of infection was 49.1% (27/55); however, if prolonged cough symptoms are taken into account, the percentage of parents who are putative transmitters of the infection to their infants increases to 56.3%. These estimates are in line with similar studies conducted in infants <6 months of age [21,135]. When information about symptoms in other family members were added, the source of transmission rose to 87.3% of the infants hospitalized for pertussis. These results are higher than those reported by other studies, and could reflect a closer pattern of household contacts in Italian families as compared to other countries. Several studies described asymptomatic parents as able to transmit pertussis to vulnerable infants. Across these studies, 8-13% of contacts who remained asymptomatic had laboratory evidence of recent pertussis infection [117,136,137]. In our study, this percentage is 23.6%.

This part of our project might be of help for the assessment of future integrated strategies, to be associated with vaccination, in order to contain pertussis circulation. The cocoon strategy, already described, might substantially reduce the disease burden of pertussis by limiting its transmission [132], but it is not sufficient. In fact, Warfel et al, have shown in a non-human primate model for *B. pertussis* infection that individuals vaccinated with current aP vaccines can become asymptomatically infected, and can then transmit infection to susceptible individuals [95]. Since the infection might pass asymptomatic, serological monitoring of the family members, and boosters to adolescents and adults should also be considered.

The most important strength of our work is that we studied a highly selected population, including very young infants the most who had not received full immunization yet. Through this study design, we were able to identify results that were completely independent from the effect of vaccination. Moreover, our laboratory diagnostic procedures for pertussis and criteria for cases enrollment were standardized.

Limitations of our study firstly, it was conducted in a small sample size and in a restricted hospital setting that would not be representative of the whole country, but also potentially biased by the selection criteria requiring hospitalization. Secondly, we failed to compare clinical manifestations in infants with different genetic profiles of *B. pertussis*, and to investigate the post-infection follow-up in terms of microbiological and immunological characterization. Furthermore, all infants were

younger than 4 months of age and healthy controls were not matched for age; we cannot exclude that a long duration of breastfeeding and the older age of controls may have enhanced the protective effect of breastfeeding after this period of life. Finally, we cannot take into account an actual exposure to *B. pertussis* in the household and the serological framework of the contacts is incomplete, being focused only on parents of the pertussis cases; moreover, we missed to obtain information on the presence of cough in the relatives of the infants in the group of control.

6. CONCLUSIONS

In conclusion, *B. pertussis* infection, classically considered an ancient disease, is resurging worldwide and remains a serious potential health risk to newborns, especially among those too young to be vaccinated, who have the highest risk of developing severe disease and complications and should be strictly monitored for the disease. The prevalence and severity of the disease among infants should increase the attention on pertussis, leading to better strategies for its prevention and care.

Breastfeeding remains a milestone of prevention for a number of diseases, including several infectious diseases. We strongly support the choice of exclusive breastfeeding that should be promoted for the first six months of life, as WHO and CDC strongly recommend. The risk of infant morbidity for acute respiratory infections is negatively associated with the duration of breastfeeding; its benefits are particularly evident in low-income settings, where a number of risk factors may add to exposure to infectious diseases. Conversely, in developed countries exclusive breastfeeding does not play a significant role in protecting unvaccinated children from pertussis and other protective strategies should be considered for preventing the disease shortly after birth.

Paediatric vaccination is no longer sufficient to prevent this threatening disease. It is mandatory to ask health care professionals to advise parents about pertussis transmission to the newborns and to be particularly aware of coughing symptoms in the household. The impact of cocoon strategy may be hampered by asymptomatic colonization and transmission from vaccinated individuals in the households. Serological monitoring of the family members should be considered to acquire evidence on the risk of pertussis transmission to the newborns.

Moreover, taking into account the potentially higher probability of infection transmission in families with frequent and long contacts among household members, we support the adoption of recommendations issued by the Center for Disease Control and Prevention for the prevention of respiratory infections transmission to infants. According to these recommendations, symptomatic mothers should thoroughly wash their hands with soap and water before touching the infant and cover their nose and mouth with a mask if symptomatic for cough in close contact with the infant.

More effective vaccines should include adjuvants able to increase the number of specific long-lived plasma cells; at the same time, monitoring the evolution of the bacteria might allow the timely detection of escape mutants, particularly the confirmation of strains not expressing the vaccine antigens [137-139]. This project supports the need for new preventive strategies and the development of new vaccines which might induce a more effective and prolonged protection should be evaluated.

7. TABLES AND FIGURES

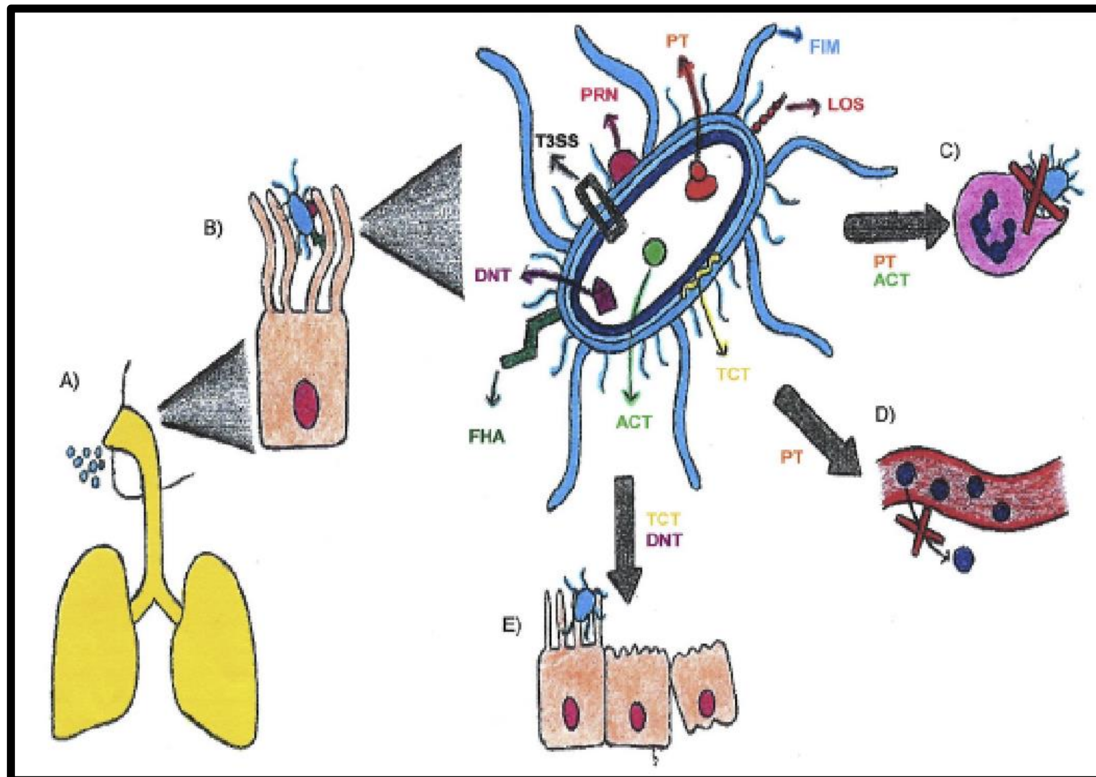


Figure 1 - Pertussis pathogenesis. *B. pertussis* is transmitted person to person through aerosolised droplets and reaches the upper respiratory tract (A), where it attaches to the ciliated cells through the fimbriae (FIM), the filamentous hemagglutinin (FHA), the pertussis toxin (PT), the lipooligosaccharide (LOS) and the pertactin (PRN). *B. pertussis* inhibits neutrophil phagocytosis with the PT and the adenylate cyclase toxin (ACT), it blocks lymphocyte migration to the site of infection with the PT and it causes ciliostasis, necrosis and extrusion of the ciliated cells through the tracheal cytotoxin (TCT) and the dermonecrotic toxin (DNT). (G. Di Mattia, et al. Paediatr Respir Rev. 2019 Feb;29:68-73)

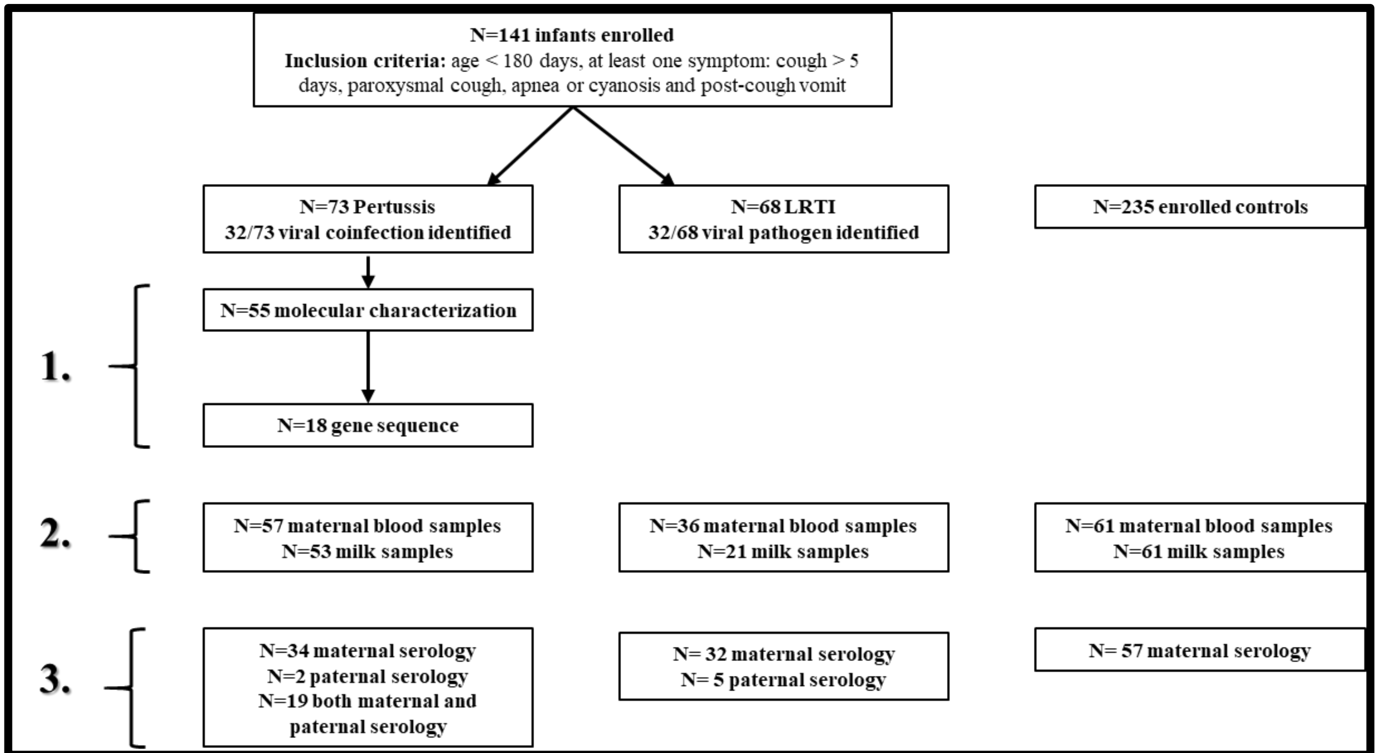


Figure 2 – Flow chart patients enrolled in the study. 1) Microbiological surveillance of pertussis and description of analysis of new variants of *B. pertussis* circulating. 2) Study of the protective role of breastfeeding and maternal immunity in whooping cough. 3) Serum-epidemiology of pertussis in parents of patients affected by whooping cough.

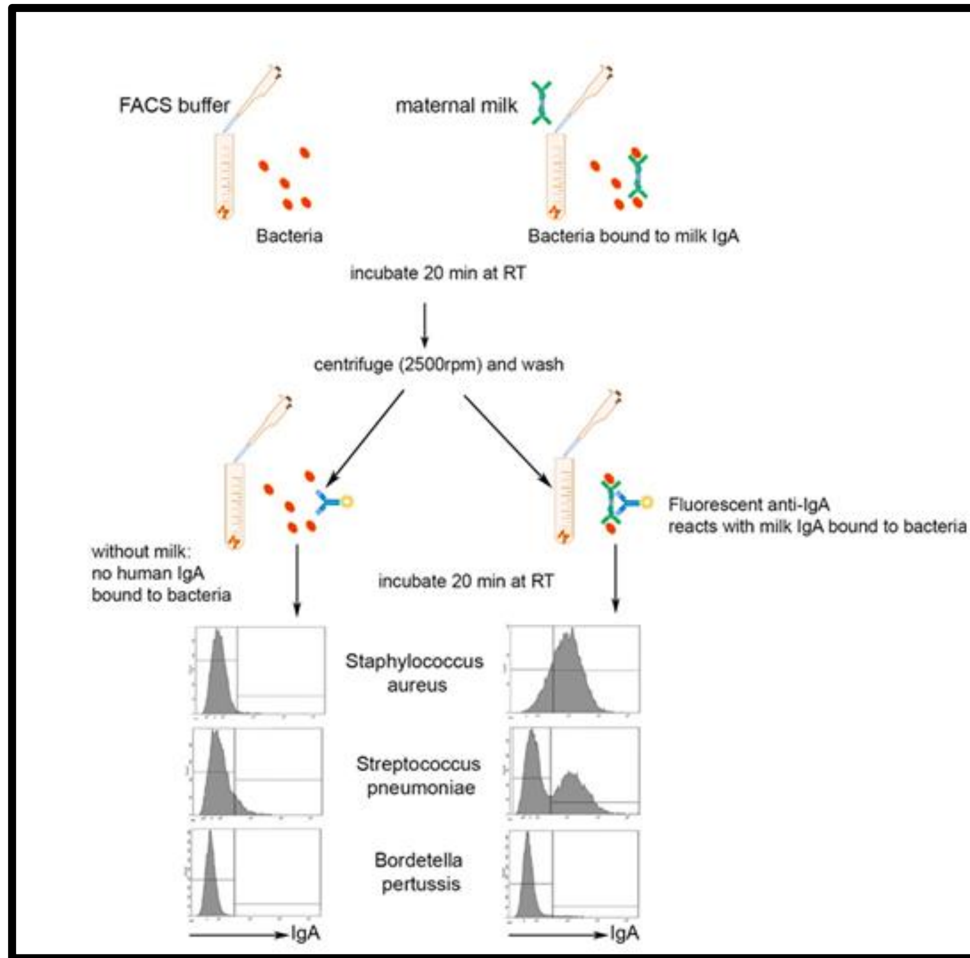


Figure 3 - Bacterial FACS method. Colonies of bacterial isolates were diluted in FACS buffer and incubated either with FACS buffer or with maternal milk (1:10 and 1:30 dilutions). After washing, IgA bound to bacterial cells was revealed by a secondary staining with FITC-labeled mouse anti-human IgA.

Demographic and clinical variables	*Pertussis N = 73*	*LRTI N = 68*	*p values*	*OR*
Age in days median (range)	45 (7-165)	49 (7-152)	ns	-
Males (%)	44 (60.2)	28 (41.1)	< 0.05	2.14
Vaccination 1 dose (%)	9 (12.3)	4 (5.8)	ns	1.13
Vaccination 2 doses (%)	3 (4.1)	0	-	-
Macrolide before admission (%)	21 (29.0)	10 (15.0)	ns	1.09
Steroid before admission (%)	31 (43.0)	16 (25.0)	ns	1.13
Days of cough at admission median (range)	10 (0-44)	4 (0-48)	ns	-
Days of cough after discharge median (range)	18 (0-100)	7 (4-30)	ns	-
Days of hospitalization median (range)	7 (1-41)	4 (0-36)	ns	-
Cough (%)	67 (92.0)	65 (96.0)	ns	0.96
Paroxysmal cough (%)	61 (84.0)	29 (44.0)	< 0.05	1.96
Post-cough vomit (%)	41 (56.0)	16 (24.0)	< 0.05	2.39
Cyanosis (%)	42 (56.0)	12 (18.0)	< 0.05	13.5
Apnea (%)	55 (75.0)	15 (23.0)	< 0.05	13.6
Fever (%)	13 (18.0)	32 (47.0)	ns	0.40

Table 1 – Demographic and clinical characteristics of the 141 infants enrolled (pertussis vs LRTI)

MLVA type	ptxA1	ptxP		prn2	
		ptxP1	ptxP3	producing	deficient
MT 27	16	0	16	7	9
MT 28	1	0	1	1	0
MT 60	1	1	0	1	0
Total	18	1	17	9	9

Table 2 – Molecular characteristics of 18 *B. pertussis* isolates

Demographic variables	Pertussis N = 61	HC N = 235	p values
Age in months mean (SD)	1.53 (0.77)	2.26 (0.65)	< 0.001
Males (%)	37 (60.7)	120 (51.1)	ns
Caucasian (%)	51 (83.6)	220 (93.6)	0.012
Gestational age in weeks mean (SD)	38.7 (1.5)	38.9 (1.6)	ns
Premature birth (%)	4 (6.6)	15 (6.4)	ns
Birth weight in Kg mean (SD)	3.260 (0.480)	3.250 (0.490)	ns
C-section (%)	21 (34.4)	95 (40.4)	ns
Employed mother (%)	34 (55.7)	142 (60.4)	ns
Employed father (%)	57 (93.4)	216 (92.7)	ns
Mother with university degree (%)	13 (21.1)	95 (40.4)	0.006
Father with university degree (%)	10 (16.7)	75 (32.3)	0.017
Adult households ≥ 3 (%)	15 (24.6)	24 (10.2)	0.003
One or more sibling (%)	39 (63.9)	104 (44.3)	0.006
Smoker mother (%)	7 (11.5)	24 (10.2)	ns
Smoker father (%)	18 (29.5)	75 (31.9)	ns
Feeding type at symptoms onset (%)			0.007
- Exclusive breastfeeding	35 (57.4)	114 (48.5)	
- Artificial feeding	8 (13.1)	77 (32.8)	
Partial breastfeeding	18 (29.5)	44 (18.7)	
Exclusive days of breastfeeding mean (SD)	31.3 (30.1)	39.7 (35.3)	ns
Never breastfed (%)	14 (23.0)	65 (27.7)	ns

Table 3– Demographic characteristics of infants enrolled (pertussis vs HC)

Covariable	OR	95% CI	p values
Breastfeeding at admission	1.21	0.31-4.67	ns
Days of breastfeeding	1.01	0.98-1.04	ns
Age in days	0.94	0.92-0.97	< 0.001
Male	1.51	0.74-3.07	ns
Caucasian	0.41	0.14-1.24	ns
Gestational age in weeks	0.87	0.66-1.15	ns
Birth weight in Kg	1.28	0.56-2.92	ns
Vaginal delivery	1.47	0.66-3.29	ns
Mother with university degree	0.45	0.15-1.31	ns
Father with university degree	0.33	0.10-1.06	0.063
Employed mother	1.28	0.58-2.82	ns
Employed father	1.61	0.37-7.07	ns
Adult households ≥ 3	2.08	0.81-5.35	ns
One or more sibling	2.55	1.21-5.35	0.013
Smoking mother	1.11	0.32-3.87	ns
Smoking father	0.59	0.27-1.31	ns

Table 4 – Effect of exclusive breastfeeding on pertussis: multivariable regression model

Immunologic Test	*Pertussis N=61* Median	Range	*HC N=235* Median	Range	*p values*
IgA PT	0.24	0.11-0.93	0.21	0.11-0.41	ns
IgA PNR	0.14	0.08-0.54	0.15	0.10-0.30	ns
IgA FHA	0.13	0.08-0.45	0.14	0.11-0.25	ns
IgA Streptococcus Pneumoniae	0.34	0.21-1.74	0.34	0.16-0.76	ns
Bacterial FACS (%)					
- ***Bordetella pertussis***	4.35	1.00-41.70	2.80	0.90-45.40	0.004
- ***Streptococcus salivaris***	11.50	1.10-38.80	16.40	0-62.20	ns
- ***Enterococcus faecalis***	8.50	0.80-51.20	8.20	0.10-53.40	ns
- ***Klebsiella pneumoniae***	4.80	0.30-58.70	5.50	0.30-46.90	ns
- ***Pseudomonas aeruginosa***	7.70	1.40-28.60	5.10	0.40-12.50	ns
- ***Escherichia coli***	3.35	0.20-20.00	2.80	0.50-81.70	ns
- ***Staphylococcus aureus***	22.20	2.10-87.20	20.70	1.00-86.30	ns
- ***Streptococcus pneumoniae***	8.60	2.20-49.00	12.30	0.70-70.30	ns
- ***Klebsiella oxytoca***	5.40	0.20-47.10	9.60	0.90-87.10	ns

Table 5 – Breast milk IgA against pertussis antigens, breast milk IgA bacterial binding (pertussis vs HC)

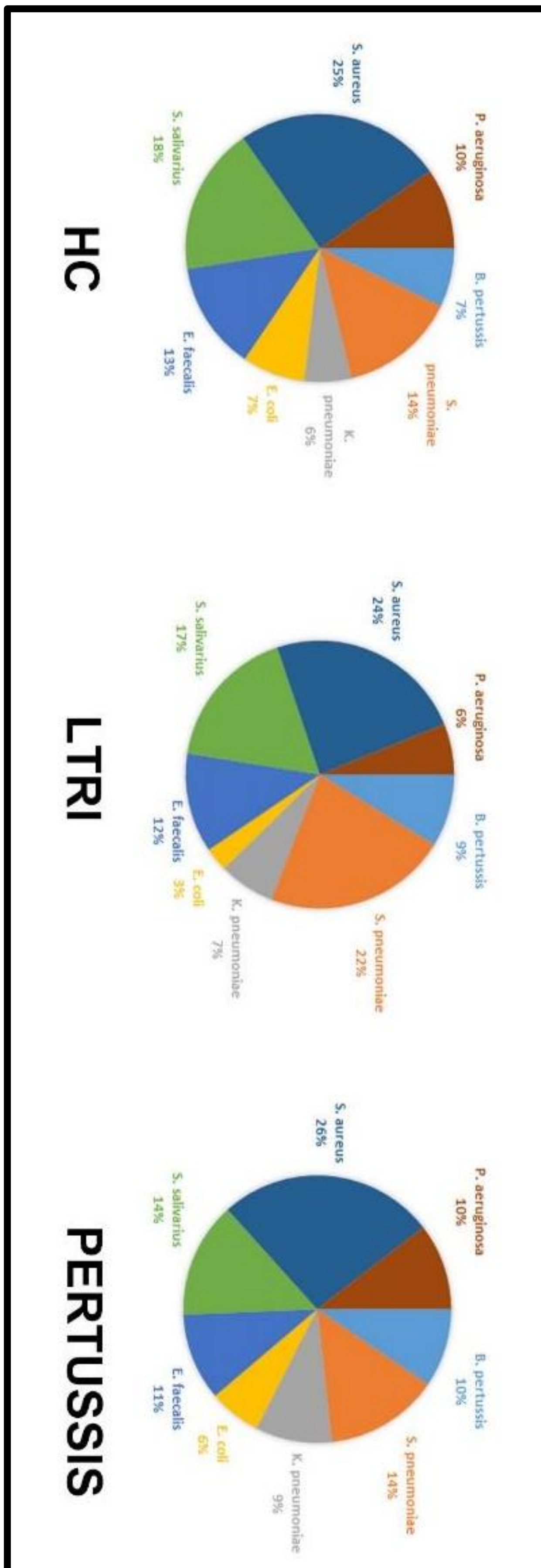


Figure 4 - Representation of IgA binding to the specific bacteria in the breast milk (HC vs LRTI vs pertussis mothers).

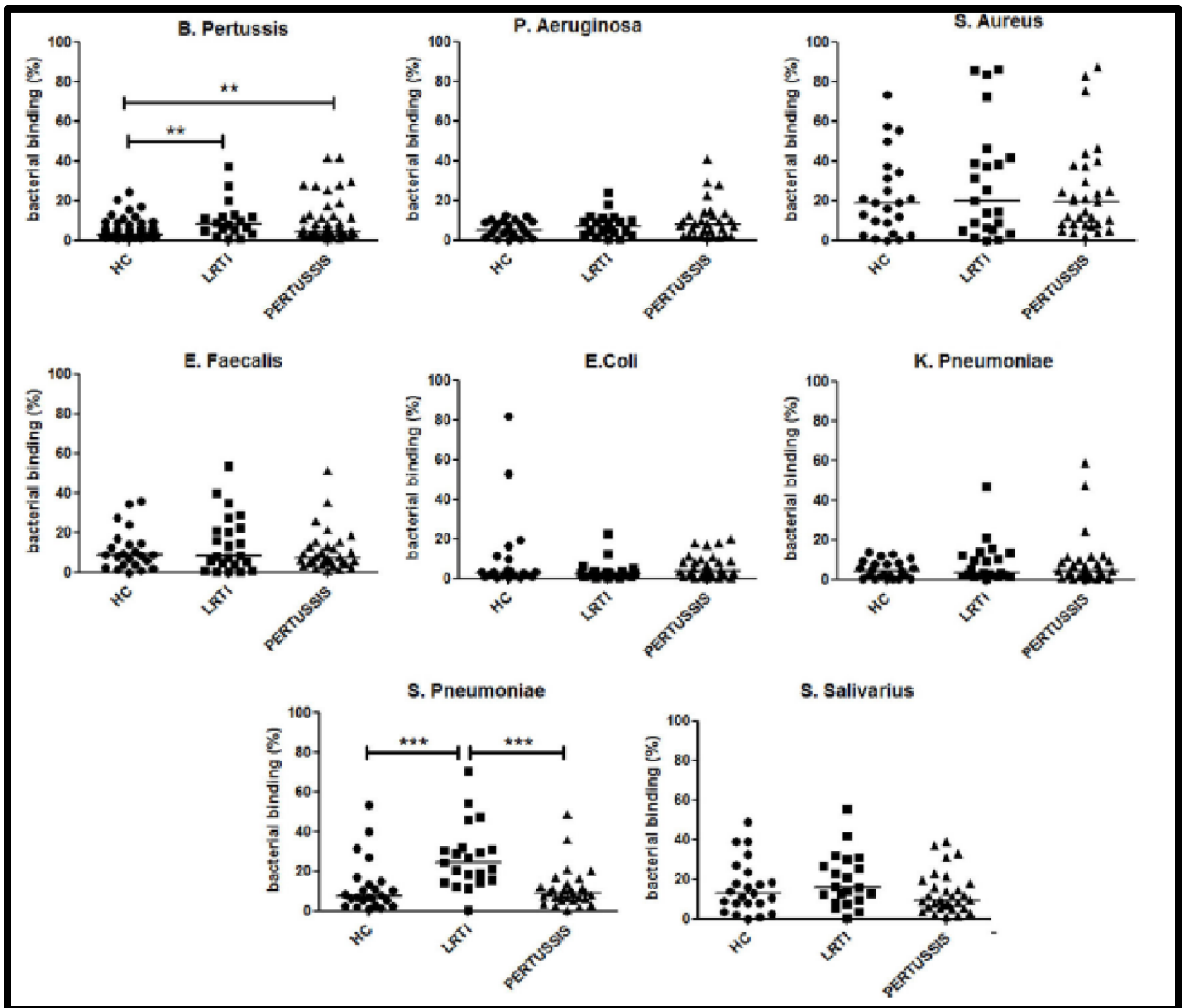


Figure 5 – Frequency of specific bacteria binding to breast milk IgA (HC vs LRTI vs pertussis mothers, $^{**}p < 0.01$ and $^{***}p < 0.001$)

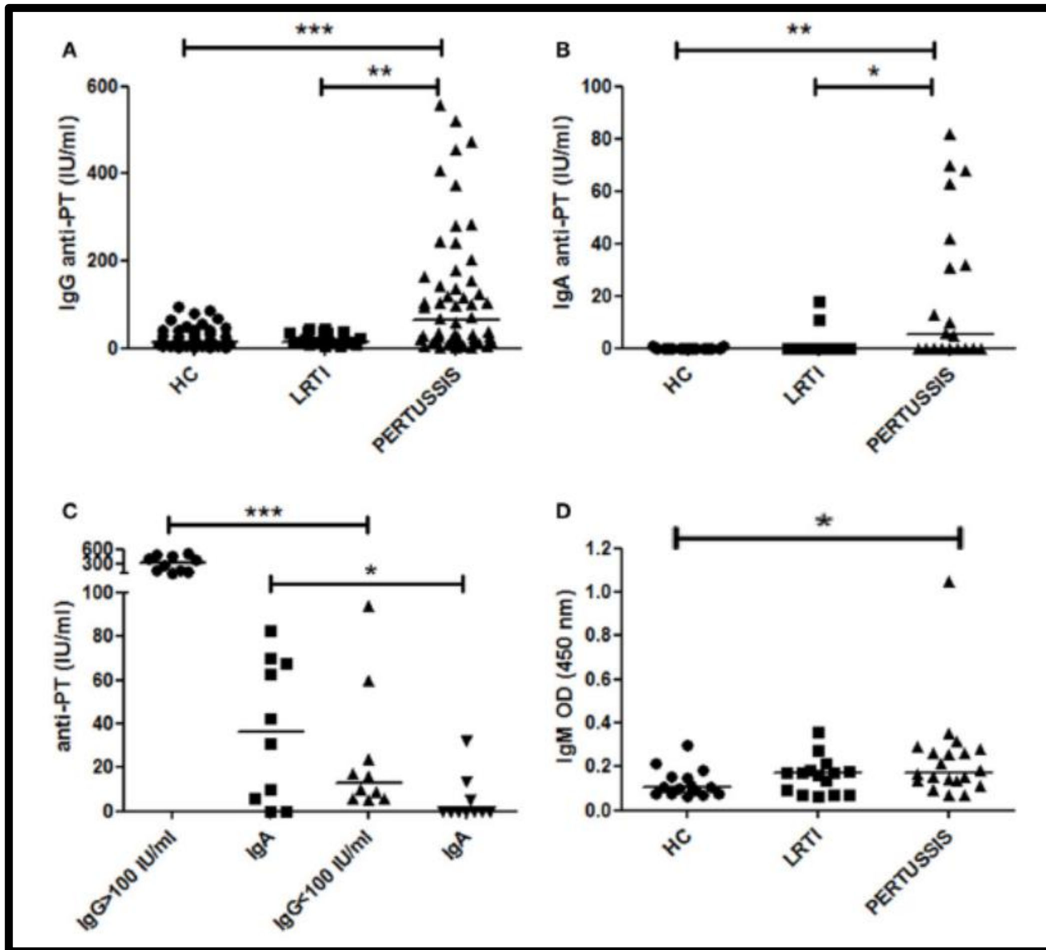


Figure 6 – Specific maternal Ig anti-PT. **(A, B, D)** Concentration of IgG anti-PT, IgA anti-PT, and IgM anti-PT (HC vs LRTI vs pertussis mothers). (**C**) Concentration of IgA anti-PT in the serum of pertussis mothers with IgG anti-PT ≥ 100 IU/mL or with IgG anti-PT < 100 IU/mL. (*p < 0.05, **p < 0.01, and ***p < 0.001)

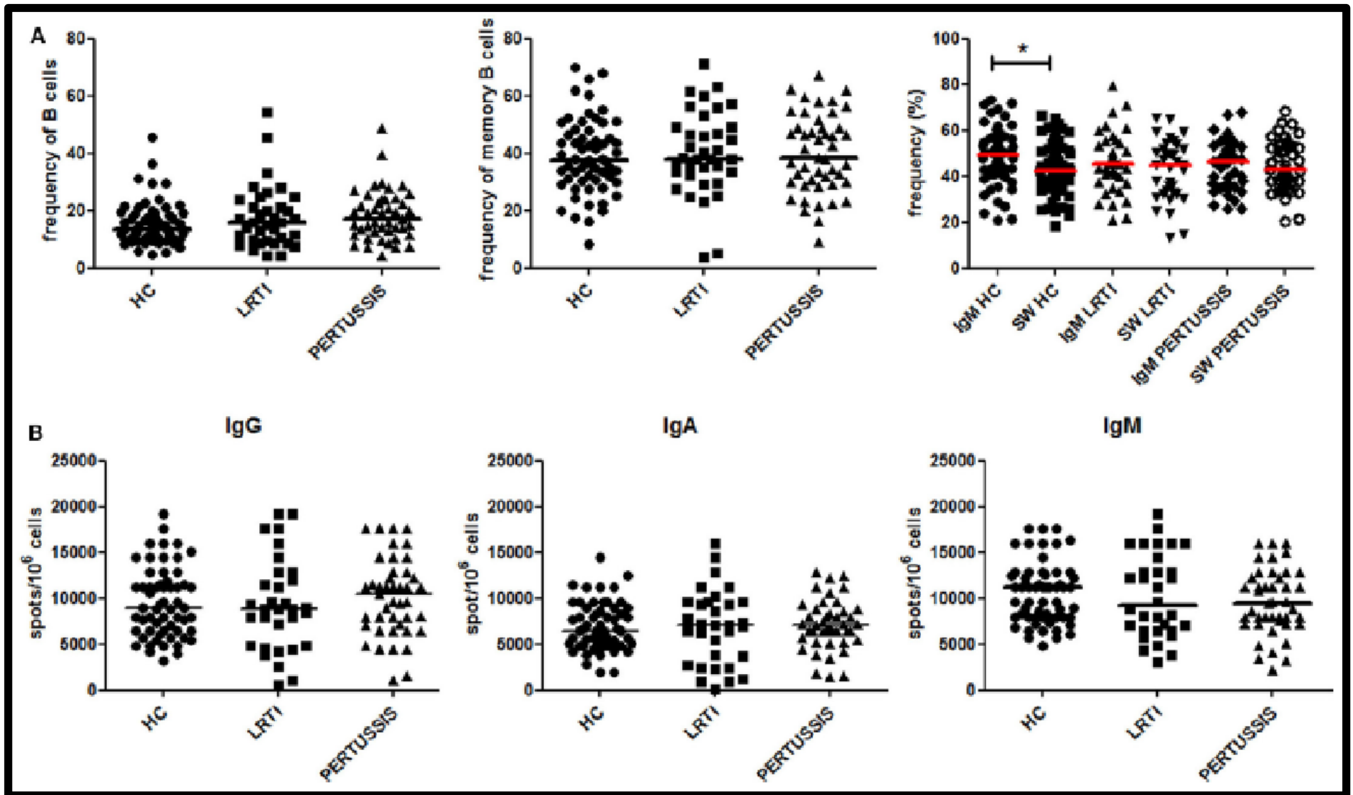
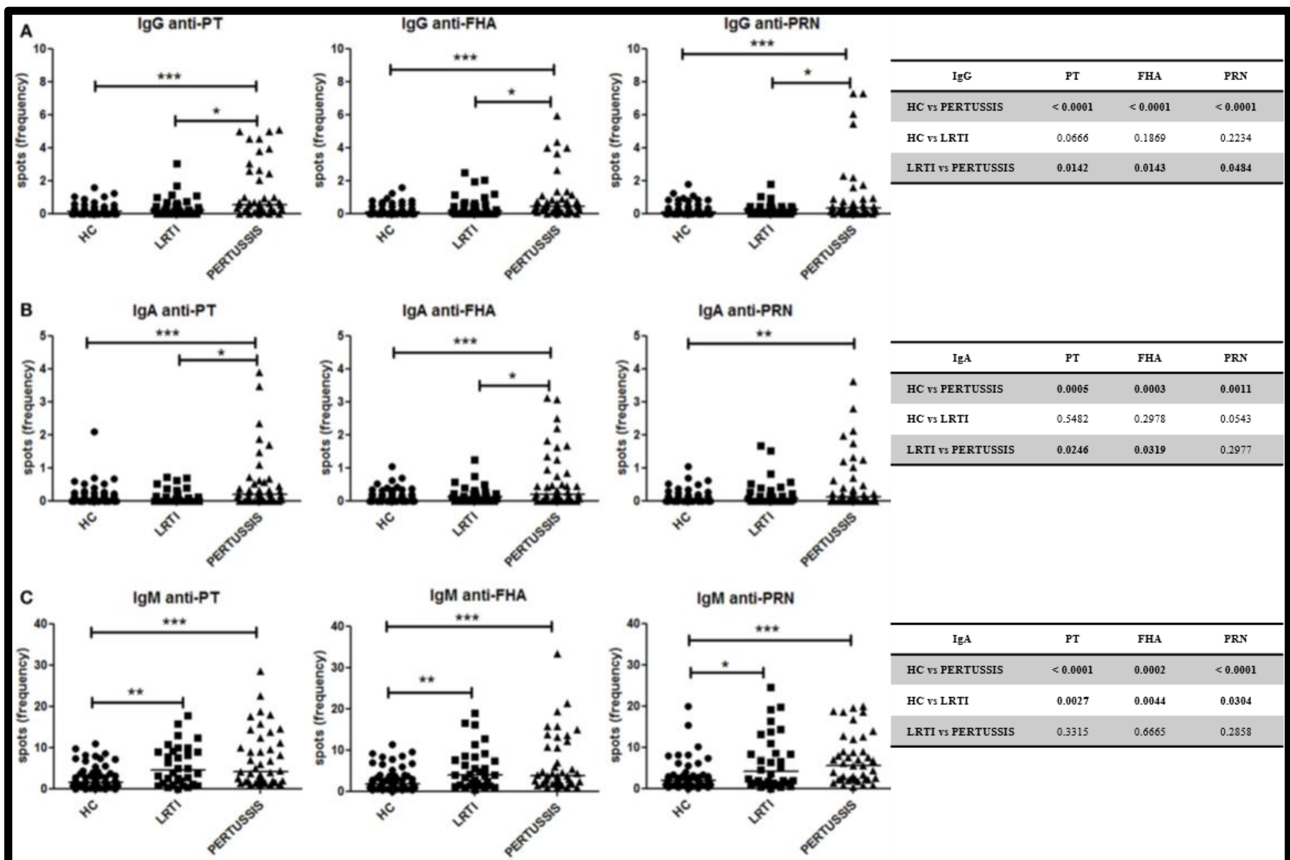


Figure 7 - Peripheral B-cell subset analysis. **(A)** Frequency (%) of B cells (defined as CD19+), total memory B cells (CD19+CD27+), IgM (CD19+CD27+IgM+), and switched (CD19+CD27+IgM−) memory B cells (HC vs LRTI vs pertussis mothers). **(B)** Total IgM, IgA, and IgG spots per million of total peripheral blood mononuclear cells (HC vs LRTI vs pertussis mothers) *p=0.014



IgG	PT	FHA	PRN
HC vs PERTUSSIS	< 0.0001	< 0.0001	< 0.0001
HC vs LRTI	0.0666	0.1869	0.2234
LRTI vs PERTUSSIS	0.0142	0.0143	0.0484

IgA	PT	FHA	PRN
HC vs PERTUSSIS	0.0005	0.0003	0.0011
HC vs LRTI	0.5482	0.2978	0.0543
LRTI vs PERTUSSIS	0.0246	0.0319	0.2977

IgA	PT	FHA	PRN
HC vs PERTUSSIS	< 0.0001	0.0002	< 0.0001
HC vs LRTI	0.0027	0.0044	0.0304
LRTI vs PERTUSSIS	0.3315	0.6665	0.2858

Figure 8 - Memory B cells specific for pertussis antigens. **(A, B, C)** Number of specific anti-PT, anti-FHA, anti-PRN -IgG, -IgA, and -IgM spots per million of total cultured peripheral blood mononuclear cells (HC vs LRTI vs pertussis mothers). *p < 0.05, **p < 0.01, and ***p < 0.001

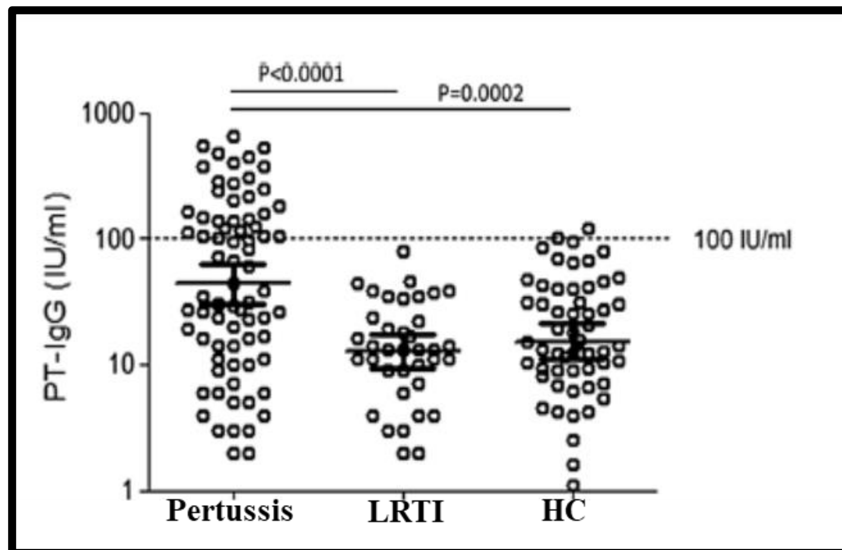


Figure 9 –IgG anti-PT serum levels in parents of infants enrolled (pertussis vs LRTI vs HC)

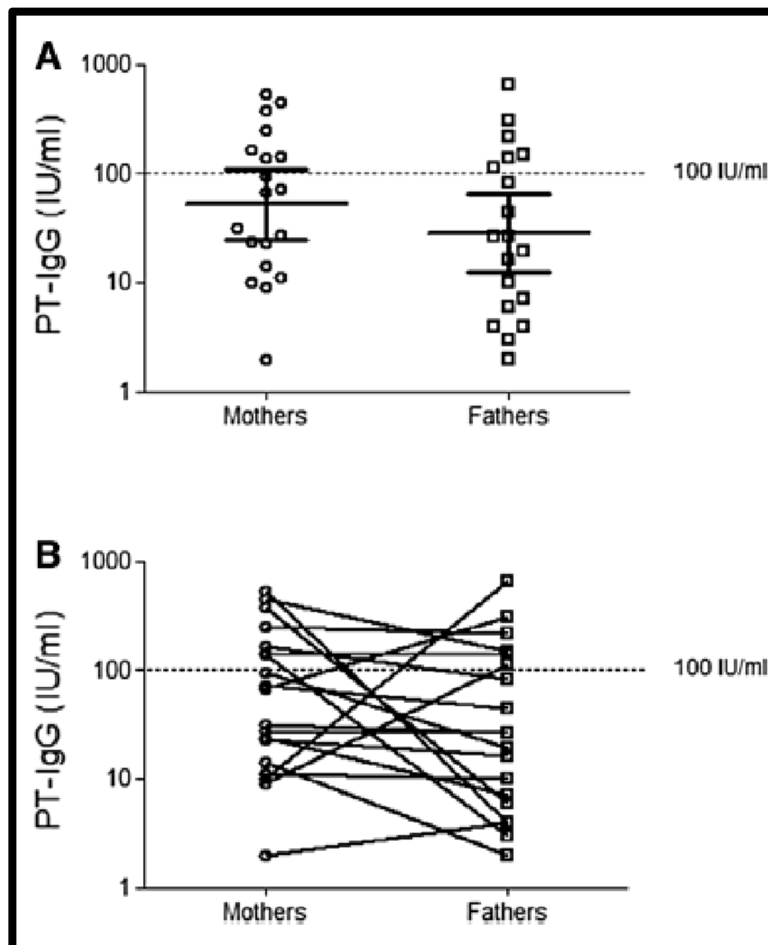


Figure 10 – Analysis of maternal vs. paternal pertussis transmission. **A)** Comparison of serum IgG anti-PT levels between mother and father of the same pertussis case. Results are expressed as IU/ml (mean and 95% CI). **B)** Plot showing the distribution of serum IgG anti-PT levels in the 19 couples who had provided a serum sample

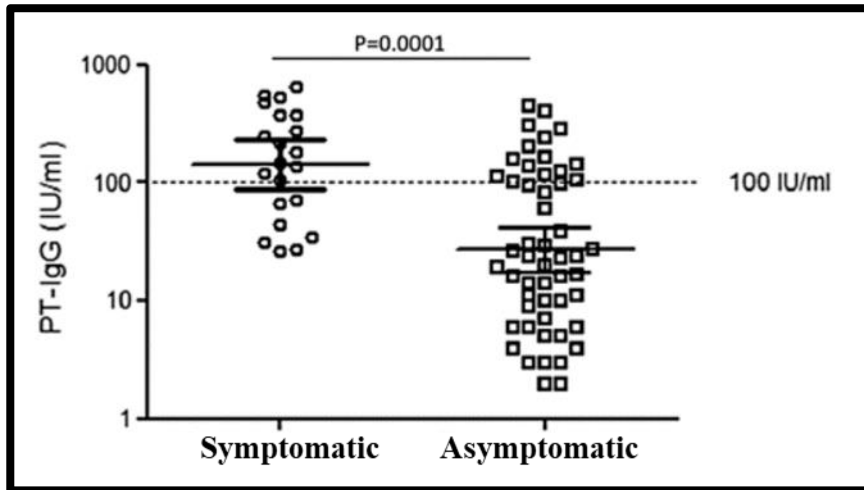


Figure 11 – Combined analysis of serology and cough symptoms in parents of pertussis infants. Plot showing serum IgG anti-PT levels in symptomatic and asymptomatic parents of pertussis infants

Parents Evaluation	*Symptomatic parent*	*Asymptomatic parent*
IgG anti-PT > 100 IU/ml (%)	14 (25.4)	13 (23.6)
IgG anti-PT < 100 IU/ml (%)	4 (7.3)	24 (43.6)

Table 6 - Evaluation of familial sources of infection. Combination of parents' serology and symptoms for single pertussis cases (N=55)

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