

RESEARCH PAPER

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Severe pertussis infection in infants less than 6 months of age: Clinical manifestations and molecular characterization

Paola Stefanelli^a, Gabriele Buttinelli^a, Paola Vacca^a, Alberto E. Tozzi^b, Fabio Midulla^c, Rita Carsetti^d, Giorgio Fedele^a, Alberto Villani^e, Carlo Concato^f, and the Pertussis Study Group[#]^aDepartment of Infectious Diseases, Istituto Superiore di Sanità, Rome, Italy; ^bMultifactorial Disease and Complex Phenotype Research Area, Bambino Gesù Children Hospital IRCCS, Rome, Italy; ^cDepartments of Pediatrics, “Sapienza” University of Rome, Rome, Italy; ^dImmunology Unit, Immunology and Pharmacotherapy Area, Bambino Gesù Children Hospital IRCCS, Rome, Italy; ^eDepartment of Pediatrics and Infectious Disease, Bambino Gesù Children Hospital IRCCS, Rome, Italy; ^fVirology Unit, Bambino Gesù Children Hospital IRCCS, Rome, Italy**ABSTRACT**

We conducted a study to determine the main traits of pertussis among unimmunized infants less than 6 months of age. From August 2012 to March 2015, 141 nasopharyngeal aspirates (NPAs) were collected from infants with respiratory symptoms attending 2 major hospitals in Rome. Clinical data were recorded and analyzed. Lab-confirmation was performed by culture and realtime PCR. *B. pertussis* virulence-associated genes (*ptxP*, *ptxA* and *prn*), together with multilocus variable-number tandem repeat analysis (MLVA), were also investigated by the sequence-based analysis on the DNAs extracted from positive samples. Antibiotic susceptibility with Etest was defined on 18 viable *B. pertussis* isolates. Samples from 73 infants resulted positives for *B. pertussis*. The median age of the patients was 45 d (range 7–165); 21 infants were treated with macrolides before hospital admission. Cough was reported for a median of 10 d before admission and 18 d after hospital discharge among infected infants, 84% of whom showed paroxysmal cough. No resistance to macrolides was detected. Molecular analysis identified MT27 as the predominant MLVA profile, combined with *ptxP3-ptxA1-prn2* associated virulence genes.

Although our data may not be generalized to the whole country, they provide evidence of disease severity among infants not vaccinated against pertussis. Moreover, genetically related *B. pertussis* strains, comprising allelic variants of virulence associated genes, were identified.

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infant; molecular characterization; pertussis; vaccination; virulence gene

Introduction

Bordetella pertussis is the etiological agent of whooping cough or pertussis. It primarily affects children less than 6 months of age with severe clinical symptoms, causing an excess of admissions to intensive care units. The most common manifestations of *B. pertussis* infection are whooping cough and bronchitis, with complications including pneumonia, seizures, encephalopathy, and possibly sudden infant death.¹

Pertussis is a vaccine-preventable disease. However, despite the large use of primary immunization, outbreaks have been reported also in countries with a high vaccination coverage.² Several reports recognized in the waning immunity and in the circulation of variants *B. pertussis* strains possible determinants of the re-emergence of pertussis.³ Moreover, the circulation among households^{4–6} could contribute to the transmission of the disease to infants too young to be vaccinated. Finally, the current risk for infant in the first months after birth and the crucial role of a pertussis booster in pregnancy may certainly be the key to address strategies to prevent infant death.



The disease is increasing due to many factors, including the circulation of variants among *B. pertussis* isolates carrying virulence associated genes different from the vaccine strains. In

particular, the promoter region of pertussis toxin (*ptxP*), the region encoding the subunit S1 of the toxin (*ptxA*) and pertactin (*prn*) genes, are the main gene targets analyzed in the circulating pertussis isolates as acellular vaccine (ACV) components.

In Europe, the European Centre for Disease Control and Prevention (ECDC)-funded network, Eupert-labnet, reported a very similar *B. pertussis* population for 12 countries using ACVs; in particular, circulating strains are distinct from vaccine strain with respect of one or more virulence associated genes.⁷

In Italy, pertussis vaccination coverage is estimated to be around 95%.⁸ Actually, the pertussis ACV is offered free of charge, being included in the hexavalent vaccine, in combination with other vaccines against diphtheria, tetanus, polio, hepatitis b and *Haemophilus influenzae* type b. An incidence rate of pertussis of 1 per 100,000 inhabitants has been reported in 2009 in the overall population in our country.⁹

Techniques for the early recognition of *B. pertussis*, such as culture or molecular methods, are not always available, thus under-diagnosis and under-reporting of pertussis often occur; rapid and accurate diagnostic tests for pertussis are needed for improved management of cases and protection especially for infants.

CONTACT Paola Stefanelli  paola.stefanelli@iss.it  Department of Infectious Diseases, Istituto Superiore di Sanità, Viale Regina Elena 299, 00161 Rome, Italy[#]Pertussis Study Group is indicated in the Acknowledgments Section.

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The main objective of our study was to estimate the proportion of infants hospitalized with suspected pertussis, defined by using the ECDC case definition,¹⁰ who are confirmed to be positive for *B. pertussis* by using appropriate laboratory diagnostic tests. Clinical symptoms and virulence associated gene variants were also evaluated among those with confirmed pertussis.

Results

A total of 73 out of the 141 enrolled children (51.8%) resulted positive for *B. pertussis*. Of the 73 confirmed cases, 75% were confirmed only by PCR, and 25% by both PCR and culture. The median age of confirmed cases was 45 d (range 7–165); of them, 12% had received one dose of vaccine, and 4% 2 doses, before the date of disease onset. A significant statistical difference ($p < 0.05$) was found between male:female ratio in infants positive or negative for *B. pertussis* (Table 1).

Paroxysmal cough was more likely reported among children with positive samples (OR 1.96; $p < 0.05$). A statistically significant OR of 2.39 for post-cough vomiting, together with cyanosis and apnea, was found for pertussis positive infants compared with the others. Other respiratory infections were detected in 32 out of 68 negative samples (47%) and in 32 out of 73 positive samples (43.8%): respiratory syncytial virus (RSV) and rhinovirus were the most frequently agents diagnosed. No other *Bordetellae* as *B. parapertussis*, *B. bronchiseptica* and *B. holmesii* were detected.

Antimicrobial susceptibility against macrolides was assessed for 18 *B. pertussis* isolates. Figure 1 showed the MIC values to azithromycin (A), clarithromycin (B), and erythromycin (C); MIC₅₀ was 0.023, 0.380 and 0.094 mg/l and MIC₉₀ 0.064, 0.500 and 0.125 mg/l, respectively.

Furthermore, 56 of the 73 positive *B. pertussis* samples, were molecularly characterized. *PtxA1* was detected in all samples.

Table 1. Demographic (A) and clinical characteristics (B) of the 141 infants with lower respiratory infection, positive or negative for pertussis lab-confirmation.

(A) Demographic Characteristics	Pertussis positive	Pertussis negative	<i>p</i> value	OR
Case n°	73	68	—	—
Age; median in days (range)	45 (7–165)	49 (7–152)	—	—
Male:Female	44/29	28/40	<0.05	2.14
Vaccination 1 dose n° (%)	9 (12)	4 (6)	>0.05	1.13
Vaccination 2 doses n° (%)	3 (4)	0 (0)	—	—
Macrolide before admission n° (%)	21 (29)	10 (15)	>0.05	1.09
Steroid before admission n° (%)	31 (43)	16 (25)	>0.05	1.13
(B) Clinical Characteristics	Pertussis positive	Pertussis negative	<i>p</i> value	OR
Case n°	73	68	—	—
Cough at admission; median in days (range)	10 (0–44)	4 (0–48)	—	—
Cough after discharge; median in days (range)	18 (0–100)	7 (4–30)	—	—
Hospitalization; median in days (range)	7 (1–41)	4 (0–36)	—	—
Cough n° (%)	67 (92)	65 (96)	>0.05	0.96
Paroxysmal cough n° (%)	61 (84)	29 (44)	<0.05	1.96
Postussive vomiting n° (%)	41 (56)	16 (24)	<0.05	2.39
Cyanosis n° (%)	42 (56)	12 (18)	<0.05	13.5
Apnea n° (%)	55 (75)	15 (23)	<0.05	13.6
Fever n° (%)	13 (18)	32 (47)	>0.05	0.4

Fifty-four samples carried *ptxP3* and 2 the *ptxP1* allele. Moreover, the pertactin variable regions¹¹ analysis revealed the presence of *prn2* allele. MLVA typing and the entire pertactin gene sequencing¹² were performed on 18 pertussis viable isolates. As shown in Table 2, MT27 profile was the predominant (16/18) and mainly combined with *ptxP3-ptxA1-prn2* genetic profile. In 9 samples it was identified the pertactin deficient gene due to the presence of the IS481 at nucleotide position 1613 or due to other modifications (data not shown).¹²

MT28 and MT60 were represented by a single isolate, associated with *ptxP3-ptxA1-prn2* and *ptxP1-ptxA1-prn2* profiles, respectively.

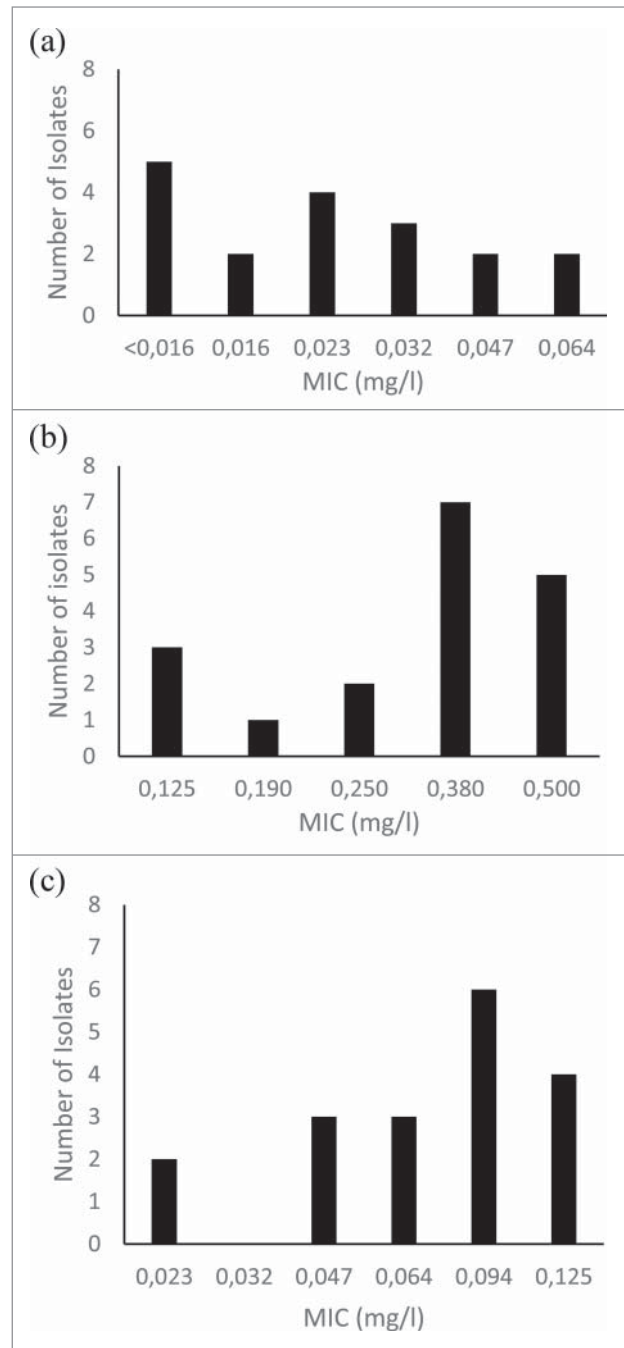


Figure 1. Distribution of Minimal Inhibitory Concentration (MIC) in mg/l for azithromycin (A), clarithromycin (B) and erythromycin (C) of 18 *Bordetella pertussis* strains.

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

MLVA Type	<i>ptxA1</i>	<i>ptxP</i>		<i>prn2</i>	
		<i>ptxP1</i>	<i>ptxP3</i>	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

Discussion

Pertussis represents a re-emergent public health issue especially for infants too young to be vaccinated. A study conducted by van Hoek¹³ in England and Wales in 2001–2011 reported 48 deaths due to pertussis, among infants under 1 y of age. When most cases occur in the first months of life, immunizing pregnant women could represent a successful strategy to protect infant by transferring maternal antibodies.¹⁴

In Italy, the vaccination against pertussis has been recommended since 1962 with the cellular pertussis vaccine. Since 1995, acellular vaccines were introduced, and in 1999 the National Immunization Plan NIP (NIP) recommended the combined DTaP (diphtheria, tetanus, and pertussis) vaccine during the first year of age, and a booster dose at 5–6 y and 11–12 y of age. At national level, pertussis vaccination coverage is high, being 94.63% in 2014.¹⁵

The diagnosis of pertussis still relies on clinical symptoms and microbiological confirmation is rarely performed in the country¹⁶; this situation determines an under-recognition and under-notification.⁹ A recent retrospective analysis by Vittucci et al¹⁷ analyzed the presence of pertussis among 215 infants observed over a 3-years period and hospitalized for any acute respiratory symptoms. Even without any specific selective clinical criteria for pertussis, the authors found 24.7% of positivity to pertussis. This finding emphasized the need in the use of laboratory confirmation tests as criterion to confirm a suspected pertussis case.

In our study, about 50% of infants with respiratory symptoms were positive for pertussis, following selective criteria of ECDC case definition. Of them, 12% received one dose of acellular vaccine; 4% had been received 2 doses of acellular vaccine, even if the second doses were received only few days before the onset of the symptoms, thus the infants were partially immunized.

Paroxysmal cough, cyanosis, and apnea were strongly associated with pertussis positivity. In particular, paroxysmal cough was more likely reported among infants with positive samples ($p < 0.05$; OR 1.96). An OR of 2.39 for post-cough vomiting, together with cyanosis and apnea, was also found among pertussis positive patients. This suggests the occurrence of severe manifestations among youngest infants with pertussis.

Respiratory syncytial virus (RSV) and rhinovirus were the most frequent co-infections associated with pertussis infection.¹⁸

Seventy-five percent of samples were lab-confirmed by PCR, and 25% by both PCR and culture, emphasizing the role of rapid diagnosis by molecular methods for a correct and prompt management of pertussis case. On the other end, there is a

need to perform simultaneously *B. pertussis* culture to evaluate the antimicrobials susceptibility and virulence associated genes, since it might play a role in understanding the differences among circulating pertussis isolates. All isolates had MICs lower than those indicated for resistance, thus macrolide and in particular clarithromycin remains the antibiotic of choice in Italy for post-exposure prophylaxis and treatment of *B. pertussis* infection.

Many studies have shown that variants have increased in frequency after the introduction of vaccines and all currently circulating strains have different genotypes from vaccine strains regarding, in particular *ptxA* and *prn* genes. The *B. pertussis* MT27 strain (double-locus variant of MT34) was the predominant type during the past decade in Australia,¹⁹ Europe,²⁰ USA,²¹ and Japan.²² As described above, the MT27 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.²⁰ It was not surprisingly that the pattern MT27 *ptxA1-ptxP3-prn2*, has been found in unvaccinated infants.²³

It is worth noting that the main molecular pattern identified was associated with severe clinical manifestations in infants. Clinical disease was not different among patients with pertactin-deficient or pertactin-producing *B. pertussis* strains as also recently demonstrated by Vodzak et al.²⁴

This study has some limitations. Firstly, it was conducted in a restricted hospital settings that would not be representative of the whole country and also potentially biased by the selection criteria requiring hospitalization. Secondly, it was not investigated the post-infection follow-up in terms of microbiological and immunological characterization. Nonetheless, our findings may contribute to describe *B. pertussis* infection in unimmunized population of small infants, underlining the need to use appropriate laboratory diagnostic tests in all infants with respiratory symptoms. *B. pertussis* virulence associated gene variants should be evaluated among those cases with laboratory confirmation.

In conclusion, *B. pertussis* infection remains a serious potential health risk to newborns, especially among those too young to be vaccinated, who should be strictly monitored for the disease. The severity of the disease among infants should increase the attention on pertussis, leading to better strategies for its prevention and care. Taken together, our data are consistent and reinforce a number of findings of other studies,^{4,18} comparing culture and molecular assay.

Methods

Specimens and patients

Nasopharyngeal aspirates were collected from infants sequentially admitted to 2 pediatric hospitals in Rome between August 2012 and March 2015. Eligible participants were children less than 6 months of age, unvaccinated or partially vaccinated with pertussis vaccine, presenting with an illness consistent with a suspected pertussis or a lower respiratory tract infection. Clinical criteria listed in the ECDC case definition,¹⁰ were used to define a suspected case of pertussis. In particular, paroxysms of coughing, inspiratory “whooping,” post-tussive vomiting, or

any person diagnosed as pertussis by a physician, or apneic episodes in infants, are included. For this analysis, clinical data were collected using a standardized and dedicated questionnaire and database. In particular, information, *i.e.*, on age, gender, pertussis vaccination status, cough at admission (presence and duration), paroxysmal cough, fever, apnea, cyanosis, was collected.

Samples were sent within few hours after collection, at room temperature, to the microbiology laboratory performing diagnosis and molecular characterization.

Bacterial culture and identification

B. pertussis cultivation was performed on Charcoal agar plates (Oxoid, England) containing defibrinated sheep blood at 10%; the plate were incubated at 37°C up to 7 d and inspected daily. 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).

Antimicrobial susceptibility assay

The minimum inhibitory concentrations (MICs) were determined using the Etest assay²⁵ for erythromycin, clarithromycin and azithromycin (MIC Test Strip, Liofilchem Italy), following standard's. Isolates with MICs of > 256 mg/l after 72 h were considered to be resistant.²⁶

Molecular diagnosis

Chromosomal DNA was extracted using QIAamp DNA minikit (QiaGEN, Hilden, Germany). The presence of *B. pertussis*, *B. parapertussis* or *B. bronchiseptica* DNA was investigated using 2 Bordetella Real Time PCR kits (Diagenode Diagnostics, Liège, Belgium and Argene, bioMérieux, Marcy l'Etoile, France); the targets are IS481 and IS1001, respectively. 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.²⁷ All Real Time PCR assay was performed using the LightCycler 2.0 (Roche Diagnostic). Data were analyzed with LightCycler software (version 4.0, Roche Diagnostic).

Molecular characterization

Sequence based typing was performed on DNAs extracted from bacterial isolates or clinical samples, using the QIAamp DNA minikit (QiaGEN, Hilden, Germany). The genes of pertactin, (*prn*), pertussis toxin promoter (*ptxP*) and pertussis toxin subunit A (*ptxA*) were sequenced using the method described previously.^{11,28} Full length pertactin gene sequencing was performed on viable isolates using primers and protocol as described previously.¹² PCR amplifications were carried out using Mastercycler personal thermal cyler (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/>).

MLVA

Multilocus variable number tandem repeat (VNTR) analysis (MLVA) 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.²⁸ 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/>).

Statistical analysis

Anonymized data were analyzed using Epiinfo software vers 3.5. Comparison was done by using Odds Ratios (OR) with 95% confidence intervals (95% CI); statistical significance was set at *p* value < 0.05.

Ethics approval

The study was reviewed and approved by the Ospedale Pediatrico Bambino Gesù Institutional Review Board (Protocol number 150 LB).

Abbreviations

ACV	acellular vaccine
ECDC	European Centre for Disease Prevention and Control
MIC	minimum inhibitory concentrations
MLVA	Multilocus variable number tandem repeat analysis
NPA	nasopharyngeal aspirate
<i>prn</i>	pertactin
<i>ptxA</i>	subunit S1 of the pertussis toxin
<i>ptxP</i>	promoter of the pertussis toxin gene

Disclosure of potential conflicts of interest statement

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