

# Detection of *Bordetella pertussis* using a PCR test in infants younger than one year old hospitalized with whooping cough in five Peruvian hospitals



María Esther Castillo<sup>a</sup>, Carlos Bada<sup>b</sup>, Olguita del Aguila<sup>c</sup>, Verónica Petrozzi-Helasvuo<sup>d</sup>, Verónica Casabona-Ore<sup>d</sup>, Isabel Reyes<sup>b</sup>, Juana del Valle-Mendoza<sup>d,\*</sup>

<sup>a</sup> Instituto Nacional de Salud del Niño, Breña, Lima, Peru

<sup>b</sup> Hospital de Emergencias Pediátricas, La Victoria, Lima, Peru

<sup>c</sup> Hospital Edgardo Rebagliati Martins, Jr, Jesús María, Lima, Peru

<sup>d</sup> Centro de Investigación de la Facultad de Ciencias de la Salud, Universidad Peruana de Ciencias Aplicadas, Av. San Marcos cuadra 2, Chorrillos, Lima, Peru

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## SUMMARY

**Objectives:** To report the incidence, epidemiology, and clinical features of *Bordetella pertussis* in Peruvian infants under 1 year old.

**Patients and methods:** A prospective cross-sectional study was conducted in five hospitals in Peru from January 2010 to July 2012. A total of 392 infants under 1 year old were admitted with a clinical diagnosis of whooping cough and tested for *B. pertussis* by PCR.

**Results:** The pertussis toxin and IS481 genes were detected in 39.54% (155/392) of the cases. Infants aged less than 3 months were the most affected, with a prevalence of 73.55% (114/155). The most common household contact was the mother, identified in 20% (31/155) of cases. Paroxysm of coughing (89.03%, 138/155), cyanosis (68.39%, 106/155), respiratory distress (67.09%, 104/155), and breastfeeding difficulties (39.35%, 61/155) were the most frequent symptoms reported.

**Conclusion:** An increase in pertussis cases has been reported in recent years in Peru, despite national immunization efforts. Surveillance with PCR for *B. pertussis* is essential, especially in infants less than 1 year old, in whom a higher rate of disease-related complications and higher mortality have been reported.

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## 1. Introduction

Pertussis is a highly contagious disease of the human respiratory tract caused by the fastidious Gram-negative coccobacillus *Bordetella pertussis*. *B. pertussis* is the primary causative agent of 'whooping cough'. It is transmitted person to person and is characterized by uncontrollable coughing fits, accompanied by inspiratory stridor.<sup>1,2</sup> This classical presentation is well-known, but has been observed less often since the implementation of immunization.<sup>3</sup>

The establishment of pertussis vaccines in the immunization schedules has reduced the global burden of disease by 90% from the pre-vaccination stage. However, the re-emergence of this disease in outbreaks has been observed around the world, both

in developed and developing countries.<sup>4–6</sup> A dramatic increase in confirmed cases in infants less than 1 year old has also been reported in recent years.<sup>1,5,6</sup> This has raised concerns, especially for infants younger than 6 months old as they are more vulnerable to disease-related complications and have a higher mortality.<sup>5,7–9</sup>

In Peru, a progressive reduction in pertussis cases was observed following national immunization efforts in 2004, and in 2010 the lowest rate of cases was reported in the last 10 years.<sup>10</sup> However, between 2011 and 2012 an abrupt increase of 20 times the incidence of pertussis cases was registered.<sup>11</sup> Furthermore, the most affected were infants under 1 year old, representing 38% of cases, despite national immunization coverage of 92% in this age group.<sup>12</sup> Currently, the whole-cell *B. pertussis* vaccine (DTwP) is the only available formulation in Peru. According to 2014 epidemiology reports, the national coverage level for this vaccination, provided in three doses as part of the pentavalent vaccine (DTwP–Hib–HepB), was 88.3%.<sup>13</sup>

\* Corresponding author. Tel.: +51 13133333.  
E-mail address: [jdelvall@upc.edu.pe](mailto:jdelvall@upc.edu.pe) (J. del Valle-Mendoza).

The disease burden of pertussis in Peru is considerable and the diagnosis is complicated by the limitations of currently available diagnostic tests. Multiple factors affect the sensitivity, specificity, and interpretation of diagnostic techniques for pertussis. Therefore, the only diagnostic tests that are accepted to confirm a case for purposes of national reporting are culture and PCR.<sup>6,8</sup> *B. pertussis* isolation by culture is the 'gold standard' and is essential for identifying the organism early in the course of disease, but has a low sensitivity with a reported range between 30% and 60%.<sup>8</sup> The DNA amplification techniques (e.g., PCR) for *B. pertussis* detection are faster and have increased the sensitivity for the overall percentage of laboratory-confirmed cases by approximately 19%, and as such represent the preferred method.<sup>14,15</sup> However, in Peru the use of PCR for surveillance was started only recently (in 2012) and there is still evidence of inadequate reporting and registration of cases, which limits the analysis of the real disease burden.<sup>12</sup>

Studies of the epidemiology of pertussis in Peru are essential in order to understand the real impact of the disease, especially following the outbreak in 2012. The aim of this study was to determine the prevalence and epidemiological and clinical characteristics of *B. pertussis* in infants less than 1 year old with suspected whooping cough in five hospitals in Peru between 2010 and 2012.

## 2. Patients and methods

### 2.1. Study population and design

A prospective cross-sectional study was conducted in five hospitals in Lima, Peru from January 2010 to July 2012: Instituto Nacional de Salud Del Niño, Hospital Edgardo Rebagliati Martin, Hospital de Emergencias Pediátricas, Hospital Nacional Cayetano Heredia, and the Hospital Regional de Cajamarca in Cajamarca. The study regions had a representative population, since Lima and Cajamarca are recognized as *B. pertussis* endemic areas and have a vaccine coverage similar to those stated in national reports.

Patients under 1 year old admitted with a probable clinical diagnosis of whooping cough were included in the study. The clinical criteria for pertussis were those given in the National Notifiable Diseases Surveillance System (NNDSS) case definition. All patients with a chronic pulmonary disease, cardiac disease, or immunodeficiency were excluded.

The project was approved by the Ethics Committee of the Hospital Nacional Edgardo Rebagliati Martins, Instituto Nacional de Salud del Niño, and Hospital de Emergencias Pediátricas in Lima, Peru. All samples were analyzed after signed informed consent was obtained from the children's parents or caregivers.

### 2.2. Samples

Nasopharyngeal samples were obtained by inserting a swab into both nostrils parallel to the palate (calcium alginate swab, USA). The swabs were placed into the same tube containing 2 ml of transport solution (PBS 1×, phosphate buffered saline). The samples were then stored at room temperature and sent to the molecular biology laboratory at Universidad Peruana de Ciencias Aplicadas (UPC). On receipt of the samples, the swabs were discarded and the tubes were centrifuged to pellet the cells, which were then resuspended in 0.8 ml of PBS 1×. One aliquot of 200 µl of each fresh specimen was used for the extraction of nucleic acids.

### 2.3. DNA extraction

DNA was extracted from a volume of 200 µl of each sample using a commercial kit (High Pure Template Preparation Kit; Roche Applied Science, Germany), according to the manufacturer's

instructions. The DNA obtained was assayed immediately or stored at −80 °C until use.

### 2.4. PCR amplification

The presence of *B. pertussis* was determined using two PCR assays, each specific for an independent region of the *B. pertussis* genome. A 191-bp fragment of the pertussis toxin S1 gene (PTxA) was amplified using the primers PTP1 5'-CCAACGCGCATGCGTG-CAGATTCGTC-3' and PTP2 5'-CCCTCTGCGTTTGTGATGGTGCC-TATTTTA-3'.<sup>16</sup> Meanwhile a 145-bp fragment of the insertion sequence IS481 was amplified using the primers IS481F 5'-GATTCAATAGGTTGTATGCATGGTT-3' and IS48R 5'-TTCAGGCAGACAACTTGATGGGCG-3'.<sup>17</sup> The procedures were modified slightly as follows: 50 µl of reaction mixture containing 25 µl Ready Mix Enzyme Solution (Taq polymerase, 2.5 mM MgCl<sub>2</sub>; 15 mM Tris/HCl PH 8.3, 50 mM KCl, 200 µM each deoxynucleotide) (Kappa Biosystems), 20 pmol of each primer (Macrogen, Seoul, Korea), water, and 5 µl DNA were amplified in a Verity Thermocycler (Applied Biosystems, Foster City, CA, USA) using a pre-denaturation step of 5 min at 95 °C, followed by 55 cycles of denaturation for 1 min at 95 °C, annealing for 1 min at 55 °C, and elongation for 45 s at 72 °C, with a final elongation of 10 min at 72 °C. The presence and size of amplification products were analyzed by electrophoresis on a 2.5% agarose gel (FMC, Rockland, ME, USA) containing 3 µg/ml of ethidium bromide in 1× Tris-borate buffer and photographed under ultraviolet illumination (UV Transilluminator KODAC LOGIC 1500, New Haven, USA). All amplified products were sequenced (Macrogen, Seoul, Korea).

Samples were determined as positive for *B. pertussis* when both the fragment of the pertussis toxin S1 gene (PTxA) and the insertion sequence IS481 were amplified, as they have been used extensively for *B. pertussis* detection.<sup>1,18,19</sup> Other fragments, such as the pertactin (prn) gene were not considered for amplification, since an increase in pertactin-deficient *B. pertussis* isolates has been reported in recent years.<sup>20</sup>

### 2.5. Statistical analysis

Qualitative variables were reported as frequencies and percentages. A seasonal index was calculated in PCR-confirmed cases for each month from January 2010 to July 2012. Seasonal indexes were calculated dividing the monthly frequency of confirmed cases by the average cases per year.

## 3. Results

A total of 392 infants under 1 year old diagnosed with whooping cough from January 2010 to July 2012 were included. The pertussis toxin and IS481 genes were detected in 39.54% (155/392) of the cases. Among all PCR-confirmed cases, infants under 3 months of age were the most affected, with a prevalence of 73.55% (114/155), and a similar sex distribution was observed. A significant number of 120 household contacts were identified by PCR, with the mother most frequently reported (20%, 31/155), followed by brothers older than 10 years old (19.35%, 30/155) and uncles (18.71%, 29/155) (Table 1).

The most common symptoms in patients with positive *B. pertussis* were paroxysm of coughing (89.03%, 138/155), cyanosis (68.39%, 106/155), respiratory distress (67.09%, 104/155), breastfeeding difficulties (39.35%, 61/155), and fever (34.19%, 53/155). In patients under 3 months of age, breastfeeding difficulties (44.7%, 51/144), apnea (21.05%, 24/144), and redness (78.07%, 89/144) were more commonly observed. Furthermore, episodes of diarrhea (17.07%, 7/41) and post-tussive emesis (60.97%, 25/41) were more frequent in infants older than 3 months of age (Table 2).

**Table 1**  
General characteristics of *Bordetella pertussis* cases

Characteristic	Total patients		Patients positive for <i>B. pertussis</i> by PCR (%)	
	Frequency (n = 392)	Prevalence (%)	Frequency (n = 155)	Prevalence (%)
Sex distribution				
Female	169	43.11	71	45.81
Male	223	56.89	84	54.19
Age				
≤3 months	266	67.86	114	73.55
>3 months	126	32.14	41	26.45
Household contacts				
Mother	63	16.07	31	20
Father	23	5.87	6	3.87
Brother ≤10 years	71	18.11	11	7.1
Brother ≥10 years	26	6.63	30	19.35
Uncle	39	9.95	29	18.71
Others	43	10.97	13	8.39
Not indicated	127	32.4	35	22.58

Complications such as acute bronchial obstructive syndrome (ABOS) and pneumonia were present in 52.22% (81/155) and 23.22% (36/155) of the cases, respectively. Intensive care unit (ICU) admission (9.03%, 14/155) and atelectasis (6.45%, 10/155) were less frequently reported complications (Table 3).

Regarding blood samples from patients with a positive PCR for *B. pertussis*, the presence of leukocytosis was reported in 24.52% (38/155) and lymphocytosis in 39.35% (61/155) of cases. Both of these laboratory abnormalities were more commonly observed in children under 3 months of age (Table 4).

A total of 10 deaths were registered during hospitalization, with nine infants being PCR-confirmed cases. The most common reported causes of death were pneumonia and sepsis. A very high leukocytosis was observed in four out of nine cases (Table 5).

Most hospitalized infants with whooping cough were admitted during the summer and autumn months (Figure 1). A seasonal index was calculated for PCR-confirmed cases during the 3 years of the study, and the highest index was registered during the period February to May, with an average between 1.19 and 1.38 (Figure 2).

#### 4. Discussion

*Bordetella pertussis* is a strict human pathogen that causes whooping cough, an endemic illness responsible for significant morbidity and mortality, especially in infants under 6 months old.<sup>1,2,5</sup> In recent years, a re-emergence of pertussis has been noted, even in countries with high vaccination coverage.<sup>4,5,21</sup> In

**Table 3**  
Complications in hospitalized infants with confirmed *Bordetella pertussis*

Complications	Total patients positive for <i>B. pertussis</i> by PCR (n = 155)		Total, n (%)
	≤3 months (n = 114)	>3 months (n = 41)	
ABOS	59 (51.75)	22 (53.66)	81 (52.22)
Pneumonia	28 (24.56)	8 (19.51)	36 (23.22)
Atelectasis	8 (7.02)	2 (4.87)	10 (6.45)
Seizures	1 (0.88)	0 (0)	1 (0.65)
Umbilical/inguinal hernia	6 (5.26)	0 (0)	6 (3.87)
ICU admission	11 (9.65)	3 (7.31)	14 (9.03)
Death	6 (5.26)	3 (7.31)	9 (5.81)

ABOS, acute bronchial obstructive syndrome; ICU, intensive care unit.

**Table 4**  
Complete blood count (CBC) in infants with *Bordetella pertussis*

Hemogram	Total patients positive for <i>B. pertussis</i> (n = 155)		Total, n (%)
	≤3 months (n = 114)	>3 months (n = 41)	
Leukocytosis ( $>18 \times 10^9/\text{mm}^3$ )	31 (27.19)	7 (17.07)	38 (24.52)
Lymphocytosis	50 (43.86)	11 (26.82)	61 (39.35)
Normal	33 (28.95)	23 (56.09)	56 (36.13)

**Table 5**  
Deaths of infants hospitalized with *Bordetella pertussis* (PCR-positive)

No.	Age, months	Leukocytes ( $10^9/\text{mm}^3$ )	Cause of death
1	1.6	28 000	Sepsis
2	2	8720	Pneumonia, sepsis
3	1	6000	Pneumonia, sepsis
4	4	ND	Sepsis
5	6	5200	Pneumonia, sepsis
6	5	6800	Pneumonia, atelectasis, ARSD
7	1	25 700	Pneumonia, myocarditis
8	< 1	44 210	Pneumonia, sepsis
9	1	39 600	Pneumonia, PH, MOD

ND, not determined; ARSD, acute respiratory distress syndrome; PH, pulmonary hypertension; MOD, multiple organ dysfunction.

Peru, an alarming increase in cases has been observed since 2011, and in 2012 an outbreak was reported in most regions, affecting primarily infants under 1 year old.<sup>10–12</sup> The most affected regions were Loreto, Ucayali, Lima, Cajamarca, Ayacucho, and Amazonas, where the highest incidences of pertussis were registered.<sup>10</sup> Thus active surveillance is required to accurately measure the disease impact on these communities, as well as vaccine efficacy.<sup>22</sup>

**Table 2**  
Clinical symptoms in PCR confirmed cases of *Bordetella pertussis*

Clinical symptoms	Patients positive for <i>B. pertussis</i> by PCR					
	≤3 months		>3 months		Total	
	Frequency (n = 114)	Prevalence (%)	Frequency (n = 41)	Prevalence (%)	Frequency (n = 155)	Prevalence (%)
Paroxysm of coughing	98	85.96	40	97.56	138	89.03
Cyanosis	79	69.2	27	65.85	106	68.39
Respiratory distress	76	66.6	28	68.29	104	67.09
Fever	35	30.7	18	43.9	53	34.19
Breastfeeding difficulties	51	44.7	10	24.39	61	39.35
Apnea	24	21.05	6	14.63	30	19.35
Ruddiness	89	78.07	24	58.54	113	72.9
Diarrhea	10	8.77	7	17.07	17	10.97
Post-tussive emesis	53	46.49	25	60.97	78	50.32
Inspiratory stridor	27	23.68	11	26.83	38	24.52
Disease onset before hospitalization:						
≤14 days	73	64.03	21	51.22	94	60.64
>14 days	35	30.70	17	41.46	53	34.19

### Frequency of confirmed and not confirmed cases of *B. Pertussis*

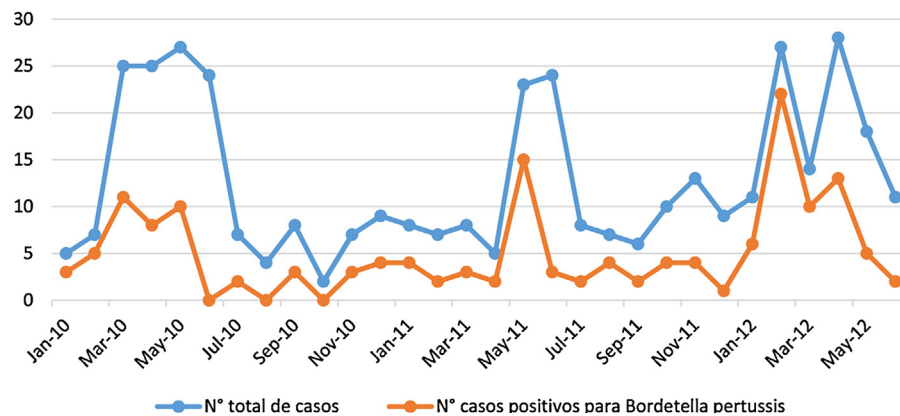


Figure 1. *Bordetella pertussis* seasonal distribution (January 2009–July 2012).

### SEASONAL INDEX

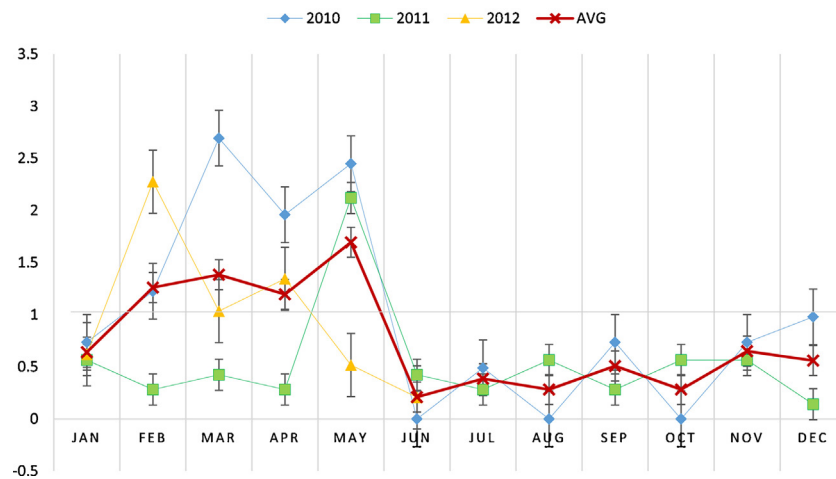


Figure 2. Seasonal index in *Bordetella pertussis* confirmed cases (January 2010–July 2012).

Evidence of a pertussis resurgence in Latin America has been demonstrated in infants less than 1 year old, and outbreaks have been observed in the last 10 years.<sup>6</sup> A study in Argentina from 2003 to 2011 reported an incidence of pertussis as high as 80.3% of cases in infants younger than 6 months old.<sup>5</sup> Another study in Panamá, performed in children under 14 years old, reported that in 2012, infants under 3 months of age represented the highest percentage of cases at 74.8%.<sup>23</sup> In Peru, a study conducted from 2003 to 2008 in infants less than 6 months of age reported that infants under 3 months old were more frequently affected in 77.6% of cases.<sup>24</sup> In the present series, 73.55% of infants with a PCR-confirmed test were under 3 months old.

The alarming increase in cases in very young infants who are unimmunized or partially immunized is of particular concern, since this age group is more vulnerable to disease-related complications and has a higher mortality.<sup>7,8,25</sup> Several factors are proposed to explain this phenomenon: post-vaccine immunity loss in household contacts, transmission from vaccinated asymptomatic individuals who remain infectious, implementation of molecular methods for diagnosis, improvements in epidemiological surveillance systems, a reduction in vaccine efficacy, and even genetic changes in the bacteria.<sup>26–29</sup>

Since most infants are infected before vaccination and concomitant protection is completed, household contacts represent an important factor in *B. pertussis* outbreaks.<sup>9</sup> Adults and adolescents have been identified as the most important sources of infection, since immunity is diminished or lost in this group and atypical clinical presentations are more common.<sup>25,26,29,30</sup> A study conducted in infants under 1 year old admitted to a pediatric ICU, identified the mothers as the most common source of pertussis infection in 50% of cases, followed by another adult in 20%, siblings in 17%, and fathers in 10%.<sup>9</sup> A previous study in infants under 6 months of age from Lima, Peru found siblings under 12 years of age to be the most frequent household contact in 40.5% of cases, followed by mothers and fathers in 36.5% and 11.7%, respectively.<sup>24</sup> In the present series, a total of 120 household contacts were identified by PCR, with the mothers also reported most frequently (20% of cases), followed by brothers older than 10 years old (19.35%) and uncles (18.71%).

The most common clinical manifestations of *B. pertussis* infection are prolonged and paroxysmal coughing, accompanied by inspiratory stridor.<sup>1,2</sup> Nevertheless, several factors are known to affect the presentation of the disease, including patient age, previous immunization or infection, presence of passively acquired antibody, and antibiotic treatment.<sup>30</sup> Multiple series have reported



paroxysmal cough (76.5–91.1%), cyanosis (46.7–81.7%), and respiratory distress (47.8–55.7%) as the most common symptoms in children.<sup>5,23,24</sup> In the present study, a similar frequency of these three symptoms was found: paroxysm of coughing in 89.03%, cyanosis in 68.39%, and respiratory distress in 67.09%. Infants under 3 months old represented 73.55% of cases, and this might explain why breastfeeding difficulties, cyanosis, apnea, and ruddiness were frequently reported.

The presence of pneumonia has been identified as the main complication in hospitalized patients with whooping cough, especially in infants under 1 year old.<sup>1,30</sup> In the USA and Canada, pneumonia is the most common cause of death and has been observed in 5.2% and 9.4% of patients, respectively.<sup>31–34</sup> Furthermore, a higher rate of cases complicated with pneumonia has been reported in Latin America, ranging from 9.0% to 25.6% of cases.<sup>23–25</sup> In the present study population, pneumonia was the second most common complication and was observed in 24.56% of infants under 3 months old.

The presence of leukocytosis and lymphocytosis has frequently been reported in confirmed cases of pertussis.<sup>23</sup> In addition, a leukocyte count  $\geq 20 \times 10^9/\text{mm}^3$  is commonly associated with pertussis infection and a count above  $30 \times 10^9/\text{mm}^3$  is an independent predictor of mortality.<sup>5</sup> In the present study, leukocytosis was reported in 24.52% of cases and lymphocytosis in 39.35% of cases. The most common causes of death reported were pneumonia and sepsis, and a very high leukocytosis was observed in four out of nine cases.

With regard to *B. pertussis* seasonality, a pattern corresponding to the summer and spring months has been reported in the southern hemisphere.<sup>5</sup> Similarly, a previous study in infants under 6 months of age performed from 2003 to 2008 in Lima, registered more hospitalizations due to whooping cough during the months of February and September. In the present study, most of the hospitalized infants were admitted during the summer and autumn months. Furthermore, a higher seasonal index was observed during the period from February to May. This pattern may differ slightly from previous reports, since this surveillance study included the 2012 outbreak in Peru.

Pertussis is an endemic vaccine-preventable disease and a major health problem in Peru. Current national vaccination strategies focus only on children and appear insufficient to prevent death in young infants. Thus, the viability of new strategies must be analyzed to include vaccination in newborns, adolescents, the elderly, health care workers, and pregnant women.

Recent evidence suggests that maternal immunization with acellular pertussis during pregnancy is safe and highly effective at protecting infants from pertussis, and that it may also have a high impact on morbidity and mortality in infants too young to have been immunized. Thus, the vaccination of pregnant women is considered likely to be the most cost-effective complementary strategy to prevent pertussis-associated infant mortality.<sup>6</sup>

Continued strengthening of pertussis surveillance in Peru is essential. In addition, the use of molecular techniques (e.g. PCR) should be encouraged for national epidemiology reporting, in order to evaluate the reliability of the data on the incidence, case-fatality, age distribution, proportion of confirmed cases, and vaccine efficacy.

In conclusion, there has been a resurgence of *B. pertussis* in Peru. The pertussis toxin and *IS481* genes were detected in 39.54% of cases presenting between January 2010 and July 2012. Infants under 3 months of age were the most affected at 73.55% of cases, and the disease was reported more commonly during the summer and autumn. The mother was the most frequent household contact, reported in 20% of cases, followed by brothers older than 10 years old in 19.35% and uncles in 18.71%. ABOS and pneumonia were present in 52.22% and 23.22% of the cases, respectively. The

case-fatality rate in the present series of patients was 5.8%, with a median age of 2.5 months.

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