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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 [a], Carlos Bada [b], Olguita del Aguila [c], Verónica Petrozzi-Helasvuo [d], Verónica Casabona-Ore [d], Isabel Reyes [b], Juana del Valle-Mendoza [d,*]

[a] *Instituto Nacional de Salud del Niño, Breña, Lima, Peru*
[b] *Hospital de Emergencias Pediátricas, La Victoria, Lima, Peru*
[c] *Hospital Edgardo Rebagliati Martins, Jr, Jesús María, Lima, Peru*
[d] *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 *IS*481 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.

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.[1,2] This classical presentation is well-known, but has been observed less often since the implementation of immunization.[3]

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.[4–6] A dramatic increase in confirmed cases in infants less than 1 year old has also been reported in recent years.[1,5,6] 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.[5,7–9]

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.[10] However, between 2011 and 2012 an abrupt increase of 20 times the incidence of pertussis cases was registered.[11] Furthermore, the most affected were infants under 1 year old, representing 38% of cases, despite national immunization coverage of 92% in this age group.[12] 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%.[13]

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

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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.[6,8] *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%.[8] 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.[14,15] 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.[12]

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′-CCCTCTGCGTTTTGATGGTGCC-TATTTTA-3′.[16] Meanwhile a 145-bp fragment of the insertion sequence *IS*481 was amplified using the primers IS481F 5′-GATTCAATAGGTTGTATGCATGGTT-3′ and *IS*48R 5′-TTCAGGCAGA-CAAACTTGATGGGCG-3′.[17] The procedures were modified slightly as follows: 50 μl of reaction mixture containing 25 μl Ready Mix Enzyme Solution (Taq polymerase, 2.5 mM $MgCl_2$; 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 *IS*481 were amplified, as they have been used extensively for *B. pertussis* detection.[1,18,19] 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.[20]

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 *IS*481 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).