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Bordetella pertussis diagnosis in children under five years of age in the Regional Hospital of Cajamarca, Northern Peru

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Abstract

Introduction: *Bordetella pertussis* is an important human pathogen that causes whooping cough (pertussis), an endemic illness responsible of significant morbidity and mortality, especially in infants and children. Worldwide, there are an estimated of 16 million cases of pertussis, resulting in about 195,000 child deaths per year. In Peru, pertussis is a major health problem that has been on the increase despite immunization efforts. The objective of this study was to determine the prevalence of *B. pertussis* among children under five years of age suspected to have whopping cough in Cajamarca, Peru.

Methodology: Children diagnosed with whooping cough admitted to the Hospital Regional de Cajamarca from August 2010 to July 2013 were included. Nasopharyngeal samples were obtained for *B. pertussis* culture and polymerase chain reaction (PCR) detection.

Results: In 133 children, the pertussis toxin and IS481 gene were detected in 38.35% (51/133) of the cases by PCR, while only 9.02% (12/133) of the *Bordetella* cultures were positive. The most frequent symptoms in patients with positive *B. pertussis* were paroxysm of coughing 68.63% (35/51), cyanosis 56.86% (29/51), respiratory distress 43.14% (22/51), and fever 39.22% (20/51). Pneumonia and acute bronchial obstructive syndrome were present in 17.65% (9/51) and 13.72% (7/51) of the cases, respectively.

Conclusions: *B. pertussis* is responsible for an important proportion of whooping cough in hospitalized children in Cajamarca. Epidemiologic surveillance programs for *B. pertussis* are essential in Peru, especially in children who could most benefit from the vaccine.

Key words: *Bordetella pertussis*; whooping cough; PCR; Peru.

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Introduction

Bordetella pertussis is an important human pathogen that causes whooping cough (pertussis), an endemic illness responsible for significant morbidity and mortality, especially in infants and children [1]. Worldwide, there are an estimated 16 million cases of pertussis, 95% of which occur in developing countries, resulting in about 195,000 child deaths per year [1,2]. Currently, an increase in reported cases of pertussis has been noted, even in countries with high vaccination coverage [3,5]. In Peru, pertussis is a major health problem that has been rising despite immunization efforts. An abrupt increase of cases was reported in 2012 and 2013, with 1,033 and 1,414 cases, respectively. Meanwhile, in previous years, the baseline number of cases reported were minor, with a total of 237 cases in 2009, 61 cases in 2010, and 89 cases in 2011 (including a total of 18 child deaths) [6]. This may be due to several factors, including the adaptation of the *B. pertussis* strains, waning vaccine-induced immunity, incomplete protection from vaccination, increased awareness, or improved reporting [5,7].

The most common clinical manifestations of *B. pertussis* infections are whooping cough and bronchitis with complications, including pneumonia, seizures, encephalopathy, and death, especially in infants with less than one year of age [1,8]. The classic

presentation of pertussis is well known, but it has been observed less often since the start of immunization [3]. Therefore, *B. pertussis* infection cannot be accurately diagnosed just by clinical presentations; laboratory tests are needed for confirmation of the diagnosis in those patients showing suggestive clinical signs and symptoms, or those with a history of exposure to infected patients [3,8,9].

Currently available *B. pertussis* diagnostic methods include culture, direct fluorescent antigen (DFA) tests, serology, and nucleic acid amplification assays such as polymerase chain reaction (PCR) [10]. Culture is specific but insensitive and time consuming [4,11]. PCR assays are faster, highly sensitive, and have shown to increase by approximately 19% the overall percentage of laboratory-confirmed cases of pertussis [9,12,13]. PCR is included in the Centers for Disease Control and Prevention (CDC) and World Health Organization (WHO) laboratory definition of confirmed cases, and its use in epidemiological surveillance has increased recently due to better capacity to measure the impact of the disease in the community, and to improved studies of vaccine efficacy [9-14].

The aim of this study was to determine the proportion of *B. pertussis* among children under five years of age suspected of having whooping cough in Cajamarca, the region with the fifth-most reported cases of *B. pertussis* in Peru.

Methodology

Patients

All patients younger than five years of age admitted to Hospital Regional de Salud de Cajamarca, in the department of Cajamarca, northern Peru, clinically diagnosed with whooping cough were included in the study. A total of 133 children were prospectively studied from August 2010 to July 2013.

Clinical and epidemiological features of each patient were recorded by a physician, including age, symptoms (paroxysm of coughing, cyanosis, respiratory distress, difficulty feeding, fever, redness, diarrhea, vomiting, apnea, inspiratory stridor, runny nose, and productive cough), days from the onset of symptoms, and time elapsed until the sample was collected and sent to the laboratory. Contact was defined as a person who presented clinical symptoms compatible with a respiratory infection in the seven days preceding the survey.

The project was approved by the ethics committee of the Hospital Nacional Edgardo Rebagliati Martins, Lima, Peru. All samples were analyzed after an informed consent was signed by the parents or caregivers of the children.

Samples

Nasopharyngeal samples were obtained by inserting a swab into both nostrils parallel to the palate (calcium alginate swab, Corona, California, USA). The swabs were placed into tubes containing 2 mL of transport solution (phosphate buffered saline [PBS] 1X). The samples were then stored at room temperature and sent to the molecular biology laboratory at Universidad Peruana de Ciencias Aplicadas Instituto de Investigación Nutricional. 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 1X. Two aliquots of 200 µL of each fresh specimen were used for the extraction of nucleic acids and 200 µL for bacterial culture.

Bacterial culture

The samples were cultured on Bordet Gengou agar (Remel, Lenexa, USA), which included 15% of sheep's blood containing 40 mg cephalexin μL^{-1} (Media Services, CPHL, Amsterdam, The Netherlands). They were incubated at 35°C in a moist atmosphere and maintained for five to seven days under aerobic conditions. The plates were visually inspected to detect any bacterial growth. Colonies were identified based on characteristic morphology, Gram stain appearance, and catalase and oxidase testing. Those suspected to be *Bordetella* spp. were tested by molecular tools as described below.

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, Mannheim, Germany) according to the manufacturer's instructions. The obtained DNA was assayed immediately or stored at -80°C until use.

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 fragment of 191 bp of the pertussis toxin S1 gene (PTxA) was amplified using the primers PTp1: 5´-CCAACGCGCATGCGTGCAGATTCGTC-3´ and PTp2: 5´-CCCTCTGCGTTTTGATGGTGCCTATTTTA- 3´ [16]. Meanwhile, a 145 bp fragment of the insertion

sequence IS481 was amplified using the primers IS481F: 5'-GATTCAATAGGTTGTATGCATGGTT-3' and IS48R: 5'-TTCAGGCAGACAAACTTGATGGGCG-3´[17]. The described procedures were slightly modified as follows: 50 µL of reaction mixture containing 25 uL ReadyMix enzyme solution (Taq polimerase, 2.5 mM Mg Cl2; 15 mM Tris/HCl pH 8.3, 50 mM KCl, 200 uM each deoxynucleotide) (Kappa Biosystems, Massachusetts, USA), 20 pmols of each primer (Macrogen, Seoul, South Korea), water, and 5 uL DNA were amplified in a Veriti Thermocycler (Applied Biosystem, Foster City, USA) using a pre-denaturation step of 5 minutes at 95°C, followed by 55 cycles of denaturation for 1 minute at 95°C, annealing for 1 minute at 55°C and elongation for 45 seconds at 72°C, with a final elongation of 10 minutes at 72°C. The presence and size of amplification products were analyzed by electrophoresis on a 2.5% agarose (FMC, Rockland, USA) gel containing 3 µg/mL of ethidium bromide in 1x Tris-borate buffer and photographed under ultraviolet illumination (UV Transilluminator KODAC LOGIC 1500, New Haven, USA). All amplified products were sequenced (Macrogen).

Statistical analysis

Qualitative variables were reported as frequencies and percentages. Comparison of proportions was determined by χ^2 or Fisher's exact test. Probability values of $p < 0.05$ were considered significant.

Results

A total of 133 children under five years of age diagnosed with whooping cough were admitted to the Hospital Regional de Cajamarca from August 2010 to July 2013. The pertussis toxin and IS481 genes were detected in 38.35% (51/133) of the cases, while only 9.02% (12/133) of the *Bordetella* cultures were positive.

Positive samples for *B. pertussis* were analyzed according to age distribution. A total of 80.39% (41/51) of cases were detected in children under one year of age. Infants younger than three months of age were the most affected group, with a prevalence of 58.82% (30/51), followed by children between one and five years of age, with a prevalence of 19.61% (10/51). Among all the positive pertussis samples, 17.65% (9/51) of patients recalled having a brother under the age of 10 with similar symptoms. Other suspected contacts were the parents in 17.64% (9/51) of the cases, followed by brothers older than 10 years of age, uncles, and cousins (Table 1).

The most common symptoms in patients with positive *B. pertussis* were paroxysm of coughing 68.63% (35/51), cyanosis 56.86% (29/51), respiratory distress 43.14% (22/51), fever 39.22% (20/51), vomiting 37.25% (19/51), and stridor 31.37% (16/51) (Table 2). Complications such as pneumonia and acute bronchial obstructive syndrome (ABOS) were present in 17.65% (9/51) and 13.72% (7/51) of the cases, respectively, and only one case of seizures was reported. Finally, 5.88% (3/51) of the children died during hospitalization (Table 3).

Table 1. General characteristics of *Bordetella pertussis* cases

Characteristics	Total patients		Patients *Bordetella pertussis* positive by PCR (%)	
	Frequency (n = 133)	Prevalence (%)	Frequency (n = 51)	Prevalence (%)
Sex distribution				
Female	61	45.86	21	41.18
Male	72	54.14	30	58.82
Age				
< 3 months	62	46.62	30	58.82
3–5 months	26	19.55	6	11.77
6–11 months	15	11.28	5	9.80
1– 5 years	30	22.55	10	19.61
Contacts				
Mother	23	17.29	4	7.84
Father	16	12.03	5	9.80
Brother ≤ 10 years of age	26	19.55	9	17.65
Brother ≥ 10 years of age	7	5.26	3	5.88
Uncle	6	4.51	3	5.88
Cousins	1	0.75	1	1.96

PCR: polymerase chain reaction

Table 2. The clinical symptoms in *B. pertussis*-positive patients

Clinical Symptoms	Total of patients		Patients positive for *Bordetella pertussis* by PCR	
	Frequency (n = 133)	Prevalence (%)	Frequency (n = 51)	Prevalence (%)
Coughing paroxysm	96	72.18	35	68.63
Cyanosis	72	54.13	29	56.86
Respiratory distress	62	46.62	22	43.14
Fever	48	36.01	20	39.22
Vomits	59	44.36	19	37.25
Stridor	42	31.58	16	31.37
Breastfeeding problems	37	27.82	11	21.57
Apnea	20	15.04	9	17.65
Redness	21	15.79	4	07.84
Diarrhea	7	5.26	2	03.92
Rinorrhea	1	0.75	1	01.96
Productive cough	1	0.75	1	01.96

PCR: polymerase chain reaction

Table 3. Complications in *B. pertussis*-positive patients

Complications	Total patients		Patients *Bordetella pertussis* positive by PCR	
	Frequency (cases)	Prevalence (%)	Frequency (cases)	Prevalence (%)
Obstructive disease	24	18.04	7	13.72
Pneumonia	23	17.29	9	17.65
Atelectasis	127	95.49	0	00.00
Seizures	3	02.26	1	01.96
Bleeding gums	1	00.75	1	01.96
Death	3	02.26	3	05.88

PCR: polymerase chain reaction

Table 4. Vaccination status in *B. pertussis*-positive patients

DPT (doses)	Total patients with DPT vaccine		Patients *Bordetella pertussis* positive by PCR	
	Frequency (cases)	Prevalence (%)	Frequency (cases)	Prevalence (%)
0	76	41.35	33	64.71
1	7	05.26	3	05.88
2	9	06.77	2	03.92
3	23	17.29	3	05.88
No data	18	29.32	10	19.61

DPT: diphtheria, tetanus, and pertussis; PCR: polymerase chain reaction

Table 5. Comparison of techniques used to identify *Bordetella pertussis*

Diagnostic method	Culture		PCR	
	Frequency (cases)	Prevalence (%)	Frequency (cases)	Prevalence (%)
Positive	12	9.02	51	38.35
Negative	121	90.98	82	61.65
Total	**133**	**100**	**133**	**100**

PCR: polymerase chain reaction

Regarding vaccination status, 64.71% (33/51) of the positive cases were unvaccinated. However, the majority of these children (30/33) were under three months of age. An unknown vaccinated status was observed in 19.61% (10/51) of patients positive for *B. pertussis*. A marked decrease was observed in children who had received at least one dose of vaccination, with a prevalence of 5.88% (3/51) (Table 4).

Differences between diagnostic method was observed, 9,02% positive samples have been detected by culture, meanwhile 38.35% were detected by PCR (Table 5).

Discussion

Pertussis is an endemic vaccine-preventable disease with the highest morbidity and mortality in the youngest infants [1,2]. In Peru, despite an overall vaccination coverage of 91%, an alarming increase of approximately 3,000% has been observed in the last years, with 1,414 new cases reported in 2013 [6,18]. The most affected regions are Loreto, Ucayali, Cajamarca, Ayacucho, and Amazonas, where the highest incidence of pertussis is registered. Thus, active surveillance is required to accurately measure the impact of the disease on these communities, as well as the efficacy of the vaccine [10].

In Cajamarca, the last epidemiological report published in 2013 reported a total of 38 cases [19]. However, in some areas of Peru, *B. pertussis* might be still underreported [3,16]. During the three-year period of our study, the incidence of *B. pertussis* in hospitalized patients with whooping cough was 9.02% (12/133) and 38.35% (51/133), determined by culture and PCR, respectively.

The use of PCR for epidemiological surveillance is increasing since it provides sensitive and rapid results. Compared with *B. pertussis* culture, PCR assays have shown to increase by approximately 19% the overall percentage of laboratory-confirmed cases [12,13,14].

In order to confirm our results, two sets of *B. pertussis*-specific PCR were used. We found an increase of 29.33% of cases with PCR compared to bacterial culture. Our results further prove that PCR is a rapid, highly sensitive, and specific method for laboratory diagnosis of pertussis. Additionally, it is important to mention that *B. pertussis* culture is a laborious process. Transport and storage of samples up to 24 hours after reception might also have played a role in reducing the positive rates of bacterial cultures, even though samples were always kept in transport medium.

B. pertussis is a highly contagious bacterium that can cause serious illness, and approximately half of infants less than one year of age who get pertussis are hospitalized. Importantly, the national immunization program of Peru includes the pentavalent vaccine (DPT-Hep B-Hib), which includes the application of three doses in less than one year in addition to the diphtheria, tetanus, and pertussis (DPT) vaccine as a booster at 18 months and four years [20]. Thus, the primary pertussis immunization series is not completed until six months of age, leaving young infants vulnerable to pertussis [1]. Our study was conducted only on hospitalized patients; this may explain the high proportion – 80.39% (41/51) – of cases found in patients under one year of age. Infants younger than three months of age were the most affected group, with an incidence of 58.82% (30/51), followed by children between one and five years of age, with an incidence of 19.61% (10/51). It is important to mention that 19.61% (10/51) of positive patients for *B. pertussis* had an unknown vaccine status, and a marked decrease was observed in children who had received at least one dose of vaccination.

Several factors known to affect the clinical manifestations of *B. pertussis* include patient's age, previous immunization or infection, presence of passively acquired antibody, and antibiotic treatment [8]. Classic illness most often occurs as a primary infection in unimmunized children, lasts 6 to 12 weeks or longer, and has three stages: catarrhal, paroxysmal, and convalescent. Common complications of classic pertussis include pneumonia, otitis media, seizures, and encephalopathy [3,8]. The most common symptoms we found in patients positive for *B. pertussis* were similar to those found in other studies, including paroxysm of coughing, cyanosis, and respiratory distress. In addition, the same symptoms occur in complications such as pneumonia and ABOS.

This study had some limitations. Only one health center was involved, non-hospitalized patients were excluded, and samples needed to be transported from Cajamarca to Lima for analysis. Therefore, we cannot rule out possible sample mishandling during any part of the process. Also, the study was designed only for *B. pertussis* detection, and the presence of other etiologies cannot be excluded.

Conclusions

As in other Latin American countries, epidemiologic surveillance for *B. pertussis* is essential in Peru, especially in children who could benefit most

from the vaccine. This study demonstrates that the incidence of *B. pertussis* is increasing in Peru and highlights the need for further investigation to better establish the impact of the disease and improve vaccination programs in children.

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Authors' contributions

JVM and JR conceived the study. VP and JVM designed the study protocol and were responsible for obtaining funding and laboratory work supervision. VC, AC performed the PCR for *Bordetella perttussi*. EC, JB, and HC were responsible for the clinical assessment, sample collection, and database completion. JVM and VC analyzed and interpreted the data. PW, JVM, MJP, and JR drafted the manuscript and critically revised the manuscript for intellectual content. All authors read and approved the final manuscript.

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