REVISTA ARGENTINA DE
MICROBIOLOGÍA

ORIGINAL ARTICLE

Bordetella pertussis, a reemerging pathogen in pediatric respiratory infections. A study in Quito, Ecuador

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Abstract *Bordetella pertussis* is the causative agent of pertussis, which mainly affects unvaccinated children, while *Bordetella parapertussis* causes a disease presenting clinical characteristics that are indistinguishable from whooping cough. Despite high vaccination coverage, pertussis remains a public health concern worldwide, with approximately 140 000 cases reported annually. Here we determined the prevalence of *B. pertussis* and *B. parapertussis* infection among infants under one year of age by polymerase chain reaction (PCR); our aim being to identify whether the data obtained relates to the relevant sociodemographic and clinical data. The study included 86 samples of nasopharyngeal swabs from infants aged between 0–12 months, who were reported as probable cases of whooping cough by the health centers around the Ecuadorian highlands, from August 2016 to July 2017. The nasopharyngeal swabs were cultured and microbiological and molecular analyses were performed. *B. pertussis* was identified by PCR in 41% of the samples (30/86), more than half of which corresponded to infants aged between 0–3 months. Moreover, a statistically significant correlation ($p < 0.05$) between the identification of bacteria in culture and the catarrhal stage of the disease was observed. The results obtained from the study highlighted the need for an active national surveillance of pertussis, in particular for laboratory testing, to provide a highly sensitive and more specific diagnosis of *Bordetella* infection.
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Bordetella pertussis, un patógeno reemergente en infecciones respiratorias pediátricas, un estudio en Quito, Ecuador

Resumen *Bordetella pertussis* es el agente causal de la tosferina, que afecta principalmente a población pediátrica no vacunada; mientras que *Bordetella parapertussis* ocasiona un cuadro clínico similar indistinguible de tosferina. A pesar de la alta cobertura de vacunación, pertussis sigue siendo un problema de salud pública a nivel mundial, con aproximadamente 140.000 casos reportados anualmente. Se determinó la prevalencia de la infección por *B. pertussis* y *B. parapertussis* en niños menores de un año, por reacción en cadena de la polimerasa (PCR), y se identificó la relación entre los datos obtenidos y los datos sociodemográficos y clínicos relevantes. El estudio incluyó 86 muestras nasofaríngeas de menores entre 0 a 12 meses de edad, quienes fueron reportados como casos probables de tosferina por centros de salud de la región Sierra de Ecuador, desde agosto de 2016 hasta julio de 2017. Las muestras nasofaríngeas fueron analizadas mediante el método de cultivo, pruebas microbiológicas y moleculares. Se identificó *B. pertussis* por PCR en el 41% de muestras (30/86), más de la mitad de estas muestras correspondieron a niños de entre 0 y tres meses de edad. Además, se identificó una correlación estadísticamente significativa ($p < 0,05$) entre la detección de las bacterias mediante cultivo y la fase catarral de la enfermedad. Con base en estos resultados, es necesario mejorar el sistema de vigilancia epidemiológica del país, principalmente, en la confirmación de casos por laboratorio, para una detección altamente sensible y específica del microorganismo causante de la tosferina.

Introduction

During the past years, a resurgence of pertussis cases has been observed worldwide and is considered a reemerging infectious disease by the World Health Organization (WHO)[26,39,46]. This disease is present in developing and developed countries, despite high rates of vaccination. It is caused by gram-negative bacteria belonging to the genus *Bordetella*, namely, *Bordetella pertussis* and *Bordetella parapertussis*, the latter causing a less severe form of the disease[3,10].

B. pertussis is known to cause more severe disease than *B. parapertussis* or *Bordetella holmesii*, while *Bordetella bronchiseptica* affects immunocompromised patients more frequently[36]. These three species of *Bordetella* share some virulence factors such as adhesins, fimbriae, filamentous hemagglutinin (FHA), pertactin (PRN), tracheal colonization factor, and tracheal cytotoxin. Conversely, pertussis toxin and serum resistance protein are expressed only by *B. pertussis*, and the type III secretion system is expressed only by *B. bronchiseptica*[37].

Among the virulence factors, it is possible to find adenylate cyclase toxin-hemolysin (CyaA), an adhesin that is frequently included as a component in acellular vaccines against *B. pertussis*[44]. Moreover, this factor is characterized by the presence of a hemolytic phenotype of virulent strains of *B. pertussis*. At present, it has been reported that the hemolytic phenotype in *Bordetella* is lost once other genes replace the toxin.

The infection has shown a modified pattern of transmission in areas with high coverage of pertussis vaccination, which is likely due to a change in circulating *B. pertussis* strains[4]. However, according to some authors, the resurgence of pertussis cases is either a consequence of bacterial host adaptation or due to vaccine influence[3]. Of the vaccines available for the prevention of the disease, the Center for Disease Control and Prevention (CDC) has recommended the use of the acellular vaccine as the primary choice for infants under one year of age, as it has been proved to be less reactogenic[10].

In Ecuador, based on the expanded immunization program (EPI), the pentavalent and trivalent whole-cell inactivated vaccines (WCV) are the pertussis vaccines being currently administered. However, in accordance with the biological drug release registry, there are also acellular pertussis vaccines (ACV) being provided by the private sector. Moreover, a decreased vaccination coverage has been observed; between 2001 and 2009 vaccination coverage was 100%; however, since 2009, this rate has been gradually decreasing[32].

Nowadays, health organizations aim at reducing the morbidity and mortality rates in lactating infants, who are under one year of age, as this population registers the majority of pertussis cases reported worldwide (88.7 out of 10 000)[41]. To achieve this objective, vaccination strategies should be evaluated; vaccination and booster doses should be implemented to cover more than 90% of the population. Moreover, epidemiological surveillance systems of the disease should be improved so that an increased number of probable cases of pertussis are identified and diagnosed[26]. In Ecuador, the Ministry of Public Health through the SIVE-Alerta program performs a passive surveillance of the disease by notifying probable cases of *B. pertussis* infection. In 2015, 7 cases were reported and in 2016 there was an increase of 14

cases[35]. Since 2016 a rapid rise in the number of cases of *B. pertussis* infection has been observed; in fact, they have duplicated in comparison to the previous year, which could be suggestive of a new outbreak. Nonetheless, the confirmation of positive cases is hampered due to a lack of highly sensitive and more specific tests, such as the polymerase chain reaction (PCR)[22,46]. This study aimed to identify the prevalence of *B. pertussis*, *B. parapertussis* and *B. holmesii* in infants under one year of age, who were reported as probable cases of whooping cough. Diagnosis was confirmed by microbiological culture and PCR, for faster and more specific laboratory diagnosis.

Methods

Population sampling

A cross-sectional study was carried out in the city of Quito, Ecuador, in coordination with the *Instituto Nacional de Investigación en Salud Pública* (INSPI). The Ecuadorian highland region was chosen for evaluation as it has reported the highest number of pertussis cases since 2016[35]. The samples to be analyzed consisted of 86 nasopharyngeal swabs from children between 0 to 12 months of age, who were described as probable cases of whooping cough after visiting the different health centers in the region.

The study was limited to a type of observational study that analyzes data from a population, from the Epidemiological Surveillance System for this disease. This study involves data collected at a defined time. The study was reviewed and approved by the Human Research Ethics Committee of the Pontificia Universidad Católica del Ecuador, which categorized it as scientifically acceptable.

Samples

Samples were received either as nasopharyngeal swabs in the Regan-Lowe Semi-solid Transport Medium (calcium alginate swab, one swab per nostril) or as nasopharyngeal aspirate specimens in a sterile bottle. The samples were analyzed by the gold standard method: microbiological culture, and by endpoint PCR. The latter followed an established protocol for the amplification of *B. pertussis* targets IS*481* and *ptxA*-Pr, *B. parapertussis* IS*1001* and *B. holmessi* hIS*1001*. This study was done from August 2016 to July 2017.

Data collection

The epidemiological chart of each sample was evaluated to retrieve relevant sociodemographic and clinical data. A database was created, including the following information for each sample: the age and gender of the patient, place of origin, type of sample, elapsed time from symptom onset to primary diagnosis, which was determined by the practitioner based on the clinical symptoms.

Microbiological culture

Nasopharyngeal swabs were cultured in Regan-Lowe medium (Becton Dickinson), containing 5% cephalexin, and were incubated in a humidity chamber at 37 °C for 7–10 days. After this period, the presumptive *Bordetella* spp. colonies were subjected to further testing to differentiate *B. pertussis* from *B. parapertussis*. Microbiological and biochemical tests were used, including Gram staining, MacConkey agar, oxidase, and catalase tests.

PCR for the detection of *B. pertussis* and *B. parapertussis*

Each swab sample was suspended in 500 μl of PBS, and genomic DNA was extracted using the High Pure PCR Template Preparation Kit (Roche Germany), according to the manufacturer's instructions.

To differentiate among the *Bordetella* species, specific primers were used for the following targets: IS*481* and *ptxA*-Pr were used for the identification of *B. pertussis*, IS*1001*, for the identification of *B. parapertussis*, and the hIS*1001* target for the identification of *B. holmesii*[22,40] (Table S1). To amplify the bacterial genome, 1 μl of the extracted DNA was added to 11.5 μl of the master mix preparation (GoTaq®) to make up a final volume of 12.5 μl. The conditions used in the thermocycler were the following: denaturation (5 min at 94 °C); annealing for IS*481* (30s at 63.6 °C); annealing for *ptxA*-Pr (30s at 55 °C); annealing for IS*1001* (30s at 60.4 °C); and annealing for hIS*1001* (30s at 63.6 °C) extension (30 s at 72 °C); final extension (10 min at 72 °C); 25 cycles were used to amplify the genes.

Statistical data analysis

Descriptive statistics were used to analyze the clinical information of the patients, and the results obtained from the microbiological cultures and PCR. The non-parametric chi-square test (χ^2) was used to compare the results obtained from the cultures and PCR to establish a diagnostic value. The parameters of sensitivity, specificity, positive predictive value and negative predictive value were also analyzed.

Results

Of the 86 samples of probable cases of whooping cough, 29 (35%) belonged to patients who were hospitalized in the pediatric unit at health centers in Quito. No significant difference was observed between genders, as 55% of patients were male, and 45% were female. The study included a population ranging from 0–12 months of age, which was distributed as follows: 59 patients were between 0–3 months old; 20, between 4–6 months old; 4, between 7–9 months old, and 3 between 10–12 months old. More than half of the samples used in the study, 62 of 86 (72%), came from patients going through the first 2 weeks since symptom onset, whereas 24 (28%) were past this time frame (Table 1). From the epidemiological charts in our possession, it was not possible to verify the vaccination status or the antimicrobial treatment of the patients; therefore, these factors, if any, were not included in our analysis.

From the samples that were analyzed, 23% (20 of 86) were found to be positive for *B. pertussis* by culturing, whereas the results obtained from PCR showed that 41% (30 of 86) of

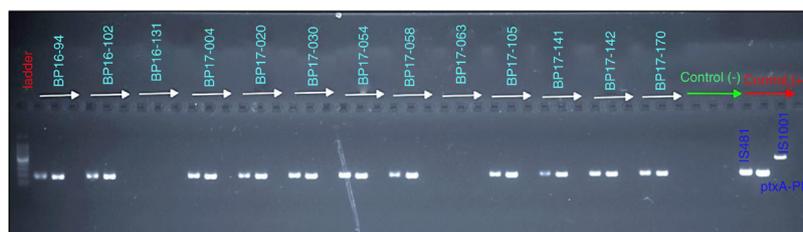**Table 1** Characteristics of the study population.

Variables	n	%
Gender		
Male	47	55
Female	39	45
Age (months)		
0–3	59	65
4–6	20	24
7–9	4	7
10–12	3	3
Onset of symptoms (days)		
1–5	9	11
6–10	32	37
11–15	21	24
16–20	14	16
21–25	10	12
Medical care		
Hospitalized	84	98
Ambulatory	2	2
Season		
Rainy (winter)	53	62
Dry (summer)	33	38
TOTAL	86	100

The data was retrieved from the epidemiological charts of patients reported as probable cases of whooping cough in Quito, Ecuador.

Figure 2 Amplification of IS*481* and IS*1001* to confirm the co-infection of *Bordetella pertussis* and *Bordetella parapertussis* (BP17-82). PCR products were analyzed in a 2% agarose gel by electrophoresis.
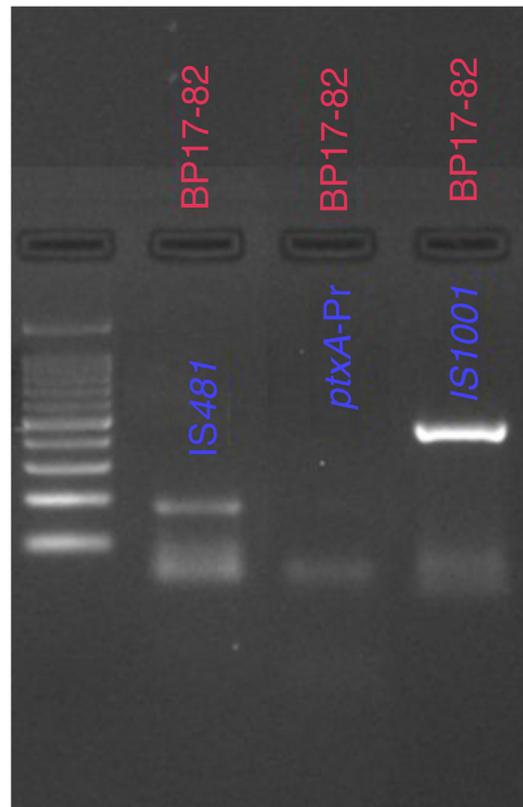
cases were positive for IS*481* and *ptxA*-Pr, which are specific to *B. pertussis* (Fig. 1). Moreover, one case was identified as a co-infection of *B. pertussis* and *B. parapertussis*, since both IS*481* and IS*1001* were amplified (Fig. 2), while the hIS*1001* did not amplify any sample (Table S2).

By comparing the techniques used for the detection of the bacteria, it was observed that the polymerase chain reaction showed a sensitivity of 79% and a specificity of 71%. In contrast, the gold standard method showed 100% specificity, and 23% sensitivity (20/86) (Table 2).

Lastly, a correlation between the different stages of the disease and an identification of the bacteria in culture was analyzed, and a statistically significant result (*p*-value <0.05) was revealed for children whose swabs were collected between 1–15 days after the onset of symptoms, otherwise known as the catarrhal stage of the disease (Table 3).

Table 2 Comparison of culture and PCR for the detection of *Bordetella pertussis*.

		Culture		
		Positive	Negative	Total
PCR	Positive	15	19	34
	Negative	4	47	51
	TOTAL	19	66	85

2×2 Contingency table: Sensitivity = (15/19) = 78.9%; Specificity = (47/66) = 71.2%; PPV = (15/44) = 44.1%; NPV = (47/51) = 92.1%.

Figure 1 Amplification of IS*481*, ptxA-Pr and IS*1001* of *Bordetella pertussis*. PCR products were analyzed in a 2% agarose gel by electrophoresis.

Table 3 Correlation between the onset of symptoms of the population under study, and the detection of *Bordetella pertussis* by culture.

Onset of symptoms (days)	Culture		
	Positive for *B. pertussis*	Negative for *B. pertussis*	Total
1–5 days	2.30%	8.10%	10.50%
6–10 days	4.70%	32.60%	37.20%
11–15 days	11.60%	12.80%	24.40%
16–20 days	2.30%	14.00%	16.30%
21–25 days	2.30%	9.30%	11.60%
Total	23.30%	76.70%	100%
Pearson's chi-squared test (χ^2)	χ^2 value: 9457; df.: 4; *p*-value: 0.04		

Discussion

Whooping cough is an endemic disease with eventual outbreaks and epidemic peaks, which manifest every 2 to 5 years[13,26]. During the last decade, Latin America has reported a resurgence of pertussis post-vaccination era, the annual incidence of the disease continues to rise, and the pertussis-related mortality rate in infants younger than 6 months old is estimated at 0.2%[9,14,38].

In our study, the majority of positive cases of *B. pertussis* infections corresponded to those infants belonging to the group of 0–3 months of age, reaching a prevalence of 65%. This percentage was similar to that identified in a study carried out in Peru, in which 70% of positive cases corresponded to infants younger than 3 months old[7] as one of the main risk factors for the disease at this age is incomplete vaccination[2,9]. Nonetheless, it is worth mentioning that neither vaccination nor natural infection confers complete, lifelong immunity to the disease[42]; in fact, a minimum of two doses should be administered for vaccination to be effective. For this reason, lactating infants under 4 months of age are at an increased risk of contracting the infection[6,42].

Recently, there has been a resurgence of cases of pertussis caused by *B. pertussis*, despite good vaccination coverage[1,25]. This resurgence has been attributed to the introduction of the acellular vaccine (ACV)[34]. However, the genomic variation of pertactin (PRN) and pertussis toxin (Ptx) of *B. pertussis* has been described since the introduction of the whole-cell vaccine (WCV)[5].

Most strains used for the production of WCV and ACV express antigens such as PtxA2 or PtxA4 and Prn1 or Prn7, whereas circulating clinical isolates have other variants, namely, PtxA1 and Prn2 or Prn335. In addition, strains of *B. pertussis* have been isolated from countries with high vaccination coverage, which have lost antigens such as filamentous hemagglutinin (FHA), pertactin (Prn), Ptx subunit A (PtxA), serotype 2 fimbriae (Fim2), serotype 3 fimbriae (Fim3), and tracheal colonization factor (TcfA)[44]. While in countries with low vaccination coverage, the emergence of new cases of pertussis has been attributed to the presence of *B. pertussis* strains that express a new allele for the pertussis toxin promoter, which enhances the production of the pertussis toxin (Ptx)[34].

To this day, strains of *B. pertussis* that do not produce Prn have been isolated mainly in countries that report pertussis epidemics. In the United States, during an outbreak in 2012, it was recorded that more than 60% of isolates did not produce Prn43. Additionally; studies have shown that *B. pertussis* strains that do not produce Prn induce a stronger pro-inflammatory response, and increase cell death after infection[23].

Given that the symptoms of the disease tend to be non-specific, clinical data are vital for the adequate diagnosis of probable cases of pertussis[31]. In this study, none of the epidemiological charts contained information about the vaccination status of the patient, mother or other family members, which is necessary to monitor vaccination compliance. Furthermore, there is an immunity window of vulnerability for infants under 2 months of age[11,19]. For this reason, the WHO considers maternal immunization as an effective strategy for the prevention of pertussis in infants, as passive immunization is reached after the transfer of maternal antibodies[4,19,25].

In this study, two techniques were used for the detection of *B. pertussis* in our samples to evaluate the efficiency of the molecular technique PCR. It was identified that culturing yielded 23% of positive cases, whereas PCR detected 41%; this variation was expected since other studies estimated that without PCR, 9% to 26% of positive cases could be missed every year[17].

The results obtained from this study have shown that the application of PCR assays for the laboratory confirmation of *B. pertussis* and *B. parapertussis*, provides increased sensitivity, as it can detect minimum amounts of bacterial DNA from a sample. Moreover, to identify and differentiate among *Bordetella* species, two assays were used, each designed to amplify species-specific DNA sequences[20,27,45]. For this reason, the CDC has recommended the use of PCR as the primary method for the detection of pertussis infection[16,30].

A statistically significant correlation was observed in our study population between the catarrhal stage of the disease, which extends from day 1 to 15 after the onset of symptoms, an identification of the bacteria in culture[26,29]. In contrast, the use of PCR as a laboratory diagnostic tool amplified the window for the detection of the microorganism; the onset of symptoms can extend from day 1 to 30, including the catarrhal stage of the disease as well as the paroxysmal stage 2.

The different methodologies applied for the identification of pertussis allowed to extend the period in which the disease was detected, in comparison to the detection time of a test used in isolation[28]. Several health organizations such as the Minnesota Department of Health suggest that the optimal time for the detection of pertussis by culture should be calculated from samples collected during the first 2 weeks since the onset of symptoms[33]. Similarly, Leber AL in 2014 observed that cultures have a sensitivity of 12–60% if the analysis is performed at the beginning of the disease (<2 weeks)[29], that is, during the catarrhal stage of the disease, when there is an increased bacterial load that favors the diagnosis by culture. Moreover, Leite et al. note that in their study the sensitivity of cultures and PCR decreased after the second week since the onset of symptoms, dropping from 70% and 80% respectively to 10% and 21%[30]. Infections caused by *B. pertussis* and *B. parapertussis* that cause a pertussis-like syndrome or co-infection might be undistinguishable[18]. For this reason, a laboratory diagnosis of the etiological agent is necessary. Recently, studies have shown that the prevalence of *B. parapertussis* infections has increased[8]; however, the pertussis vaccine provides little to no protection against *B. parapertussis* infection. Based on this fact, the information obtained from this research will be made available to healthcare professionals, so that decisions are made in the clinical-epidemiological setting by taking into account the frequencies identified here for the first time.

The reemergence of pertussis is a public health concern that poses a significant challenge to existing health policies. This emphasizes that there is an atypical clinical diagnosis, as the disease manifests itself differently depending on the age, stage of the disease, immunological status, or region where it is located; these factors, therefore, complicate the epidemiological surveillance of the disease in Ecuador.

In this study, we analyzed the effectiveness of laboratory diagnostic tools for the identification of pertussis. It was observed that the use of PCR was more sensitive for the detection of the bacteria than culturing, probably because it can detect and amplify a single copy of bacterial DNA[21,24]. It is therefore likely that the negative results yielded by culture might have been false-negatives, which further supports the increased sensitivity of PCR (79%) over the cultures[12,15]. Finally, the results from the study suggest the incorporation of PCR testing as a tool for early detection of whooping cough in hospitals and epidemiological surveillance in Ecuador. We provided a rapid and accurate diagnosis of the disease to reduce the time response for the treatment of the infection, especially for infants under three months of age, considering that clinical suspicion alone is often not enough to accurately diagnose the disease[43,45,46].

Ethics approval

This study has been reviewed and approved by the Ethics Committee of Human Research of the PUCE, considering it scientifically acceptable.

Conflict of interest

The authors declare that they have no conflicts of interest.

Acknowledgments

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ram.2020.07.001.

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