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High circulation of pertussis in infants and close contacts in Antananarivo, the capital of Madagascar in Africa, and Cambodia in Asia

Gaelle Noel[1†], Aina Harimanana[2†], Laurence Borand[3†], Florence Campana[4], Chanthy Leng[3], Chhunly Botr[5], Lala Rafetrarivony[6], Mahdi Rajabizadeh[4], Alexandra Kerleguer[5], Bunnet Dim[3], Anny M. Randriamoramanana[2], Mohand Ait-Ahmed[7], Nicole Guiso[1], Jean-Marc Collard[6], Fabien Taieb[1,4*] and PERILIC working group

Abstract

Background Reliable data on whooping cough, a highly contagious disease sometimes fatal for infants, are largely lacking in low- and middle-income countries.

Methods We conducted a hospital-based prospective study (PS) on infants, and a household contact-case investigation (CCI) for positive cases throughout Cambodia and in the city of Antananarivo, Madagascar, between 2017 and 2019. The PS, in which Bordetella diagnostics (qPCR) were performed, included infants aged ≤6 months presenting with ≥5 days of cough associated with one pertussis-like symptom. CCI was performed using qPCR and serology regardless of clinical signs.

Results In this study, 207 and 173 participants from Cambodia and Antananarivo were respectively enrolled. Respectively 26.1% (54/207) and 22.0% (38/173) of the infants were infected in the cohorts from Cambodia and Antananarivo. Cough longer than 10 days appeared as a risk factor in both countries, as well as coughing spells, apnea and normal pulmonary auscultation, having a coughing contact in Cambodia. In Antananarivo, being clinically well between coughing spells appeared as a risk factor. Five infants, all positive, died during the study. In Cambodia and Antananarivo respectively, 50.9% (118/232) and 67.8% (82/121) of the contact cases were positive. Respectively 94.4% (51/54) and 90.3% (28/31) of the households had at least one positive contact case.

Conclusion The data show that pertussis circulates at high levels among infants and in their households both in Cambodia and in Antananarivo. Given the vulnerability of youngest infants, who are too young to receive fully primary vaccination, they need to be protected through boosters breaking transmission chains. Molecular diagnosis, as well as trained medical human resources to detect the disease early, are absolutely key to protect populations.

Keywords Whooping cough, Low- and -middle income countries, Risk factors, Vaccine, PCR, Serology

[†]Gaelle Noel, Aina Harimanana and Laurence Borand contributed equally to this work.

*Correspondence:
Fabien Taieb
fabien.taieb@pasteur.fr
Full list of author information is available at the end of the article

Background

Pertussis, or whooping cough, is a highly contagious respiratory illness caused by *Bordetella pertussis (Bp)* and *Bordetella parapertussis (Bpp)* and remains a great concern worldwide despite global vaccination. In a modelling study, the World Health Organization (WHO) estimated that there were 24.1 million pertussis cases and 160,700 deaths from pertussis in children younger than 5 years in 2014 [1]. National surveillance is generally the norm in high-income countries (HICs) but is largely lacking in low- and middle-income countries (LMICs) [2, 3], although estimates suggest that ninety percent of cases are in these countries [4].

Absence of laboratory diagnosis platforms, difficulty in clinically identifying the illness, and lack of pertussis awareness among the general population, medical professionals and decision makers contribute to the weakness of national surveillance and to the high number of cases in LMICs [3, 5]. As for vaccination, whole-cell pertussis vaccines (wPVs) developed in the 1940s have varying efficacy and effectiveness across producers [6], in contrast with acellular pertussis vaccines (aPVs) developed in the 1980s, whose production and quality control are more standardized and which can be administered as boosters after 6 years of age. There are important differences between the situation in most LMICs and HICs. In LMICs, the Expanded Program on Immunization (EPI) provides wPVs, unlike in HICs where aPVs are predominantly used. The immunization schedule is often restricted to three doses during the first year of life due to economic limitations in LMICs, while booster doses are administered in addition to primo-vaccination in HICs, following WHO recommendations, to limit the transmission of pertussis by children, adolescents and adults to infants who are too young to be vaccinated [7] and in whom pertussis causes a prolonged cough that can be fatal.

In Madagascar and Cambodia, the EPI vaccination schedule includes three injections using wPVs at six, ten and fourteen weeks of age, and national surveillance relies on a passive notification system based on WHO clinical definition. Recent studies have highlighted the significant circulation of *Bp* in both countries among 3–15 year olds vaccinated during their first year of life, surely due to loss of vaccine-induced immunity over time [8, 9]. This finding stands in contrast with the low annual number of cases notified in those countries over the past ten years, ranging from none to 413 and none to 54 in Madagascar and Cambodia respectively [10]. This gap suggests that the disease burden is grossly underestimated, hindering the optimal use of resources and the development of vaccination strategies tailored to the local epidemiology of the disease.

We present the results of a hospital-based prospective study (PS) linked to a contact-case investigation (CCI) made possible by the initiation of PCR and serology laboratories at the Institut Pasteur of Madagascar (IPM) and the Institut Pasteur of Cambodia (IPC). In the PS, infants under six months of age presenting with pertussis-like syndrome were included to estimate the proportion of whooping cough microbiologically confirmed by PCR. We also evaluated vaccination coverage (the proportion of eligible, vaccinated individuals) and compliance in these populations. The CCI (PCR and serology) was conducted within the households of identified index cases to assess pertussis circulation.

Materials and methods

Study population and design

The PS was conducted in Antananarivo city, Madagascar from March 2017 to April 2019, and in six provinces of Cambodia from May 2017 to July 2019. In Madagascar, infants were recruited in two university hospitals in Antananarivo. In Cambodia, recruitment was implemented in eight hospitals and clinics across six different provinces. Infants were eligible for enrollment as index cases if they were younger than six months and presenting with a persistent cough for a minimum of five days associated with at least one symptom among apnea, inspiratory "whooping" or post-coughing vomiting, or with a confirmed case of whooping cough in the entourage. After inclusion, one nasopharyngeal swab (NPS) in each nostril was collected for PCR analysis. Data on age, sex, size and weight, birth information (weight and term at birth), pertussis vaccination history based on vaccination booklets, type and duration of pertussis-related symptoms, laboratory test results (if available), antibiotic treatment type and duration, previous consultations, family composition and cases of cough in the entourage were recorded. Pertussis-related symptoms, antibiotic treatment, hospitalization and disease outcome were followed up, either when the infant was discharged from hospital or during a later visitat hospital. Individuals for whom post-tussive vomiting was reported had the 'Vomiting' variable coded as present.

For each positive (index) case, up to six contact cases, living or not in the same household, were recruited within fourteen days of respective index case inclusion. The inclusion criterion for close contacts was regular contact (> one hour/day) with the index case for at least five days before symptom onset in the infant. Upon enrollment of contact cases, one NPS in each nostril and one capillary blood sample were collected. Data on age, gender, the relationship with the index case, pertussis vaccination history based on vaccination documents or

declarative answers, and type and duration of past and current pertussis-related symptoms were recorded.

Data were collected on standardized paper questionnaires and saved in a computer database (Supplementary Fig. 2). This questionnaire was already used in the study described in [11]. In Madagascar, data were recorded by two different data clerks using a double-entry method.

Biological sample collection and analysis

All analyses (PCR and serology) were performed by the IPM and IPC laboratories, respectively. To ensure the quality and accuracy of results, double validation was conducted and, when the results were inconsistent between the IPM or IPC and the Institut Pasteur, the test was repeated.

Real-time quantitative Polymerase Chain Reaction (qPCR)

As previously described [11], samples were collected by nurses in a standardized manner across the study sites. NPS samples were taken using eSwab-containing Amies transport medium (Dacron, ref. 482CE). Samples were sent (temperature- and time-controlled transport) to the Molecular Biology Platforms at the IPM and IPC.

Quality control samples and *Bp*, *Bpp* and *B. holmesii* positive control samples were provided by Quality Control for Molecular Diagnostics (QCMD, Glasgow, Scotland), an independent International External Quality Assessment organization. Quality controls were blind tested before the study began.At laboratories level, NPS were kept at 4°C before DNA extraction within 48 hours. When not possible, the sample was stored at −80°C until processing. Samples were homogenized by vortex and the swab was discarded. Total DNA was extracted from 100 µl of the transport media (pure sample), which was then diluted: 10 µl of the transport media were spiked into 90 µl of sterile water (diluted sample) using the commercial High Pure PCR Template Preparation Kit (Roche). For each sample, both pure and diluted DNA extract solutions were tested by qPCR to address possible PCR inhibition: the diluted DNA extract solution was meant to detect PCR inhibition in the pure sample caused by too large quantity of DNA, to avoid false negative qPCR results. PCR was carried out using the commercial LightCycler 480 Probes Master kit (Roche), 0.5 µM forward and reverse primers and 0.2 µM TaqMan probe (TIB Molbiol), 2.5% DMSO (Sigma) and 5 µl of DNA extract solution. The sequence of Rnase P human gene was amplified to ascertain the quality of sampling and laboratory procedures. The amplification of insertion elements IS481 (*Bordetella spp.*) and IS1001 (*Bpp*) was first assessed. IS481 qPCR is very sensitive but not specific as it targets the DNA of both *B. pertussis* and *B. holmesii* [11]. Therefore, IS481 + samples were tested for ptxP, IS1002 and hIS1001 amplification to identify *Bp* (ptxP + and/or IS1002+) and *B. Holmesii* (hIS1001+). In cases of IS481 + samples with neither IS1002 nor hIS1001 amplification, we considered the sample as "Bor+" (Supplementary Figure S1). PCR analyses were carried out using the LightCycler 96 Instrument (Roche) and, after validation, CFX96 (Bio-Rad). Purified DNA from *Bordetella* reference strains and non-template control samples (PCR-grade water) were included as positive and negative qPCR control samples, respectively, in each qPCR.

Serology analysis

One blood sample (200–400 µl) was also collected in SST microtainer tubes from a fingertip using a 23G lancet needle (Becton Dickinson, ref. 365968 and 369523, respectively). Once received by the laboratories, the blood was immediately spun and the serum was stored at −20°C for further analysis.

As previously described [8, 12, 13], anti-PT IgG titers were quantified using a commercial purified PT-containing enzyme-linked immunosorbent assay (ELISA) kit (EUROIMMUN; reference EI 2050-G) [14] and the WHO reference serum available from the National Institute for Biological Standards and Control (NIBSC, newly MHRA). All tests had internal negative and positive controls and passed the validity criteria. The results were reported as International Units (IU)/ml. The lower limit of quantitation (LLOQ) was defined as 5 IU/ml. Anti-PT IgG levels ≥ 40 IU/ml were considered seropositive.

Case definition

Definition of *B. pertussis* and *B. parapertussis* cases

A cycle threshold (Ct) value at 35 was considered for IS481 + PCR. Among IS481 + samples, *Bp* was molecularly confirmed with IS1002 + and/or *ptxP*+. For an individual identified as a Bor + case (only IS481+), *Bp* diagnosis was considered due to epidemiologic reasons when, the *Bp* species was found in at least one contact case by serology or PCR. If no contact fulfilled these conditions, cases were considered as negative case as well as *B. holmesii* cases in the main analysis.

Bpp was molecularly confirmed with IS1001+.

Statistical analyses

For continuous variables, medians, interquartile ranges [IQR], minimums and maximums were calculated. For categorical variables, proportions were computed and comparisons made using the two-sided Chi-square or Fisher's exact test whenever the number of observations in any category was below five; the non-parametric Wilcoxon Rank Sum test was used for continuous variables. When necessary, continuous variables were categorized based on median values or values commonly

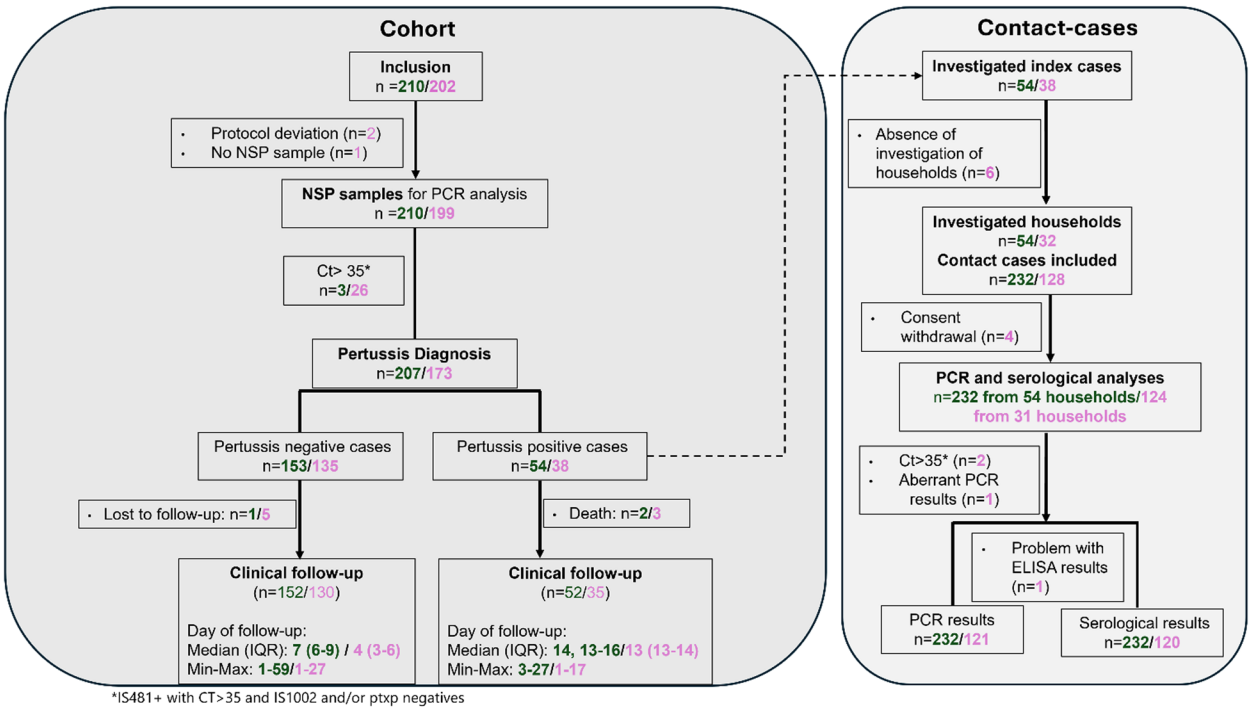

Fig. 1 Flowchart of the cohort study and contact cases investigation study. Numbers for Cambodia are written in dark green (left) and numbers for Antananarivo are represented in pink (right)

acknowledged in the literature. Spearman's coefficient was used to assess rank correlation.

In the PS, anthropometric z-scores for weight-for-height, height-for-age and Body Mass Index (BMI) for age were calculated and interpreted using the WHO and Centers for Disease Control and Prevention Child Growth Standards [15]. Weight-for-height, height-for-age and BMI were interpreted as abnormally low when the corresponding z-score was more than two standard deviations below the WHO Child Growth Standards median. The scores were computed using the zscorer package in R version 4.3.1. Data on infants' weight and term at birth were obtained from their health booklets. Data from infant hematological examinations were considered when generated up to three days before or after inclusion. We created a variable where observations made either before inclusion (reports by parents) or at inclusion (observations by doctors) were considered, so that if a sign had been reported at or before inclusion or both, it was considered as a reported clinical sign.

To define the timeliness of pertussis immunization, and due to the population of patients recruited (aged ≤ 6 months) in the PS, we assessed compliance as the number of doses received by an individual relative to the number of doses expected at their age among eligible children (i.e. older than 6 weeks). Following the pertussis vaccination schedule in Cambodia and Madagascar, timeliness with the national schedule was defined as having received the first injection at ≥ 38 days and before 10 weeks of life, and the second and third injections 17 days to 5 weeks after the previous dose. Vaccine doses were considered as received and active only when injected at least seven days before inclusion.

To evaluate the risk factors associated with whooping cough infection (identified through a positive PCR), the factors associated with the outcome with a p-value ≤ 0.20 in the univariate analysis were considered in the multivariate analysis by logistic regression. Data from infant hematological examinations were not included in this analysis due to the small sample size in Madagascar. Severity of the multicollinearity was checked through the Variance Inflation Factor (VIF) test. Only variables with VIFs below ten were included in the logistic regression model.

Statistical analyses were performed using Stata 18.0 (StataCorp LLC, Texas, USA).

Results

Hospital-based prospective study in Cambodia

Study population

A total of 210 infants were enrolled over a period of 25 months. PCR analyses were carried out on all participants. Three were excluded from the further analysis due to a Ct value above 35 for IS481 (Fig. 1).

Table 1 Population description, *p*values correspond to comparison between the 'Positive cases' and 'Negative cases' columns

Population	Antananarivo Positive cases n=38	Antananarivo Negative cases n=135	Antananarivo Total n=173	Antananarivo p-value	Cambodia Positive cases n=54	Cambodia Negative cases n=153	Cambodia Total n=207	Cambodia p-value
Gender, n (%)				0.22				0.14
Girls	20 (52.6)	56 (41.5)	76 (43.9)		30 (55.6)	67 (43.8)	97 (46.9)	
Age (months)								
Median [IQR]	1.9 (1.3-2.7)	2.2 [1.4-2.9]	2.1 [1.4-2.9]	0.32	1.9 [1.4-2.6]	2.7 [1.6-3.7]	2.5 [1.6-3.5]	**0.01**
< 3 months, n (%)	29 (76.3)	104 (77.0)	133 (76.9)	0.923	45 (83.3)	96 (62.7)	141 (68.1)	**0.01**
≥ 3 months, n (%)	9 (23.7)	31 (23.0)	40 (23.1)		9 (16.7)	57 (37.3)	66 (31.9)	
Vaccination								
DTPCV* injections				0.77				**<0.01**
0-1	31 (81.6)	102 (75.6)	133 (76.9)		50 (92.6)	93 (60.8)	143 (69.1)	
2-3	3 (7.9)	15 (11.1)	18 (10.4)		4 (7.4)	41 (26.8)	45 (21.7)	
Declaratives & missing values	4 (10.5)	18 (13.3)	22 (12.7)		0 (0.0)	19 (12.4)	19 (9.2)	
EPI Compliance (age≥ 6 weeks)	n=28	n=104	n=132	0.43	n=43	n=119	n=162	0.69
Yes	20 (71.4)	64 (61.5)	84 (63.6)		33 (76.7)	75 (63.0)	108 (66.7)	
Declaratives & missing values	4 (14.3)	18 (17.3)	22 (16.7)		0 (0.0)	17 (14.3)	17 (10.5)	
DTPCV* injection (age≥ 6 weeks)								**<0.01**
0-1	21 (75.0)	71 68.3)	92 (69.7)	0.76	39 (90.7)	61 (51.3)	100 (61.7)	
2-3	3 (10.7)	15 (14.4)	18 (13.6)		4 (9.3)	41 (34.5)	45 (27.8)	
Declaratives & missing values	4 (14.3)	18 (17.3)	22 (16.7)		0 (0.0)	17 (14.3)	17 (10.5)	
Infant's morphological characteristics								
Low birth weight, n (%)				0.27				0.79
Yes	6 (15.8)	28 (20.7)	34 (19.7)		6 (11.1)	15 (9.8)	21 (10.1)	
missing values	1 (2.6)	23 (17.0)	24 (13.9)		1 (1.9)	3 (2.0)	4 (1.9)	
Weight-for-height				0.76				0.97
Median [IQR]	-.56 [-1.04_0.73]	-.45 [-1.67_0.86]	-.51 [-1.53_0.84]		-.695 [-1.49_0.7]	-.48 [-1.73_0.55]	-.625 [-1.59_0.58]	
min-max	-4.54_3.98	-6.27_3.4	-6.27_3.98		-3.56_3.28	-6.8_9.08	-6.8_9.08	
Low weight-for-height, n (%)				0.20				0.30
Yes	3 (7.9)	25 (18.5)	28 (16.2)		8 (14.8)	32 (20.9)	40 (19.3)	
missing values	8 (21.1)	24 (17.8)	32 (18.5)		0 (0.0)	3 (2.0)	3 (1.4)	
Height-for-age				0.56				0.72
Median [IQR]	-2.135 [-2.81_-1.52]	-1.78 [-3.01_-.98]	-1.88 [-3.01_-1.05]		-.725 [-1.77_0.45]	-.775 [-2.22_0.49]	-.775 [-2.14_0.46]	
min-max	-5_8.05	-5.87_1.61	-5.87_8.05		-3.86_3.81	-8.24_11.09	-8.24_11.09	
Low height-for-age, n (%)				0.62				0.32
Yes	16 (42.1)	54 (40.0)	70 (40.5)		12 (22.2)	44 (28.8)	56 (27.1)	
missing values	8 (21.1)	23 (17.0)	31 (17.9)		0 (0.0)	3 (2.0)	3 (1.4)	
BMI for age								
Median [IQR]	-1.58 [-2.24 _ 0.57]	-1.18 [-2.51 _ -0.01]	-1.25 [-2.49 _ 0.0]	0.93	-1.12 [-1.63 _ -0.07]	-.68 [-2 _0.25]	-.85 [-1.83 _ 0.25]	0.64
min-max	-5.78 _ 2.46	-6.14 _ 2.35	-6.14 _ 2.46		-2.93 _ 2.01	-5.05 _ 13.45	-5.05 _ 13.45	
Low BMI for age, n (%)								
Yes	9 (23.7)	35 (25.9)	44 (25.4)	0.81	8 (14.8)	39 (25.5)	47 (22.7)	0.11
missing values	7 (18.4)	23 (17.0)	30 (17.3)		--	--	--	--
Antibiotic therapy before inclusion								
Number of consultations before inclusion								
0	6 (15.8)	40 (29.6)	46 (26.6)	ref	11 (20.4)	28 (18.3)	39 (18.8)	ref
1	10 (26.3)	56 (41.5)	66 (38.2)	0.754	18 (33.3)	84 (54.9)	102 (49.3)	0.169
2 or more	21 (55.3)	39 (28.9)	60 (34.7)	**0.013**	25 (46.3)	41 (26.8)	66 (31.9)	0.314
missing values	1 (2.6)	0 (0.0)	1 (0.6)	--	0 (0.0)	0 (0.0)	0 (0.0)	--
Antibiotherapy, n (%)				0.61				0.40
Yes	31 (81.6)	108 (80.0)	139 (80.3)		33 (61.1)	87 (56.9)	120 (58.0)	
*Macrolides exposure, n (%)** [duration]*	17 (54.8) [13<72h, 4>72h]	44 (40.7) [30<72h, 14>72h]	61 (43.9) [43<72h, 18>72h]	0.16	4 (12.1) [4<72h]	15 (17.2) [13<72h, 2>72h]	19 (15.8) [17<72h, 2>72h]	0.59
missing values	1 (2.6)	0 (0.0)	1 (0.6)		12 (22.2)	32 (20.9)	44 (21.3)	
Duration of cough at inclusion								
median (days) [IQR]	12 [7-16]	7 [6-10]	7 [6-11]	**<0.01**	9 [6-12]	7 [6-9]	7 [6-10]	**<0.01**
min-max	5-55	5-52	5-55		5-30	5-36	5-36	
Duration of cough ≥ 7 days, n (%)	30 (78.9)	78 (57.8)	108 (62.4)	**0.02**	40 (74.1)	84 (54.9)	124 (59.9)	**0.01**
Duration of cough ≥ 14 days, n (%)	13 (34.2)	15 (11.1)	28 (16.2)	**<0.01**	11 (20.4)	10 (6.5)	21 (10.1)	**<0.01**

*DTP-containing vaccines (Diphtheria, Tetanus and Pertussis)

**Percentage among patients having received antibiotic therapy

Among the 207 participants, the gender ratio (M/F) was 1.13 and the median [IQR] age was 2.5 [1.6–3.5] months, ranging from 13 days to 6.4 months. The population description is shown in Table 1. Briefly, 143 (69.1%) infants received fewer than two vaccine injections against pertussis. 108 of the 162 (66.7%) infants above 6 weeks of age were compliant with the EPI.

Birth weights were low for 21 (10.1%) infants. As described in the Table 1, before inclusion 168 (81.2%) infants had previously attended at least one medical consultation. Hundred and twenty (58.0%) had received antibiotic therapy. Among them, macrolides had been given to 19 (15.8%), and 13 infants (10.8%) had received antibiotic therapy independently of any previous consultation. As described in the Supplementary Table 1, regarding clinical symptoms, apnea, cyanosis, inspiratory whoop and post-tussive vomiting were observed before inclusion for 32 (15.5%), 71 (34.3%), 80 (38.6%) and 114 (55.1%) patients, respectively.

Regarding the infants' household characteristics, the median [IQR] number of people per household was 6 [4–7] ranging from 3 to 17, including 3 [2–4] adults ranging from 2 to 10. The median [IQR] number of people between 6 and 15 years old was 0 [0–1], ranging from 0 to 9 children. The median [IQR] number of people between 0 and 5 years old was 1 [1–2], ranging from 0 to 7. Cough in the entourage was reported by 127 (61.4%) individuals.

As described in the Table 1, at inclusion, the median [IQR] duration of cough was 7 [6–10] days, ranging from 5 to 36 days. Twenty-one (10.1%) infants presented with

Table 2 Symptomatology, when reports before and at inclusion are considered together (pink: Antananativo: green: Cambodia)

Clinical assessment at and before inclusion	Madagascar Positive cases n=38	Madagascar Negative cases n=135	Madagascar Total n=173	Madagascar p-value	Cambodia Positive cases n=54	Cambodia Negative cases n=153	Cambodia Total n=207	Cambodia p-value
Coughing spells, n (%)	34 (89.5)	119 (88.1)	153 (88.4)	> 0.9	43 (79.6)	88 (57.5)	131 (63.3)	**<0.01**
Apnea, n (%)	5 (13.2)	22 (16.3)	27 (15.6)	0.64	23 (42.6)	25 (16.3)	48 (23.2)	**<0.01**
Cyanosis, n (%)	15 (39.5)	54 (40.0)	69 (39.9)	> 0.99	33 (61.1)	62 (40.5)	95 (45.9)	**0.01**
Missing values	0 (0.0)	1 (0.7)	1 (0.6)	--	--	--	--	--
Inspiratory whoop, n (%)	9 (23.7)	29 (21.5)	38 (22.0)	0.86	31 (57.4)	71 (46.4)	102 (49.3)	0.18
Missing values	0 (0.0)	5 (3.7)	5 (2.9)	--	0 (0.0)	1 (0.7)	1 (0.5)	--
Difficulty in breathing, n (%)	35 (92.1)	128 (94.8)	163 (94.2)	0.46	43 (79.6)	121 (79.1)	164 (79.2)	0.93
Vomiting, n (%)*	20 (52.6)	52 (38.5)	72 (41.6)	0.12	41 (75.9)	120 (78.4)	161 (77.8)	0.70
Post-tussive vomiting, n (%)	18 (47.4)	46 (34.1)	64 (37.0)	0.13	40 (74.1)	114 (74.5)	154 (74.4)	0.95
Good condition between cough, n (%)	32 (84.2)	102 (75.6)	134 (77.5)	0.26	47 (87.0)	122 (79.7)	169 (81.6)	0.23

*Patients with post-tussive vomiting are considered as having vomited as well

Table 3 Data from hematological examinations. *P*-values correspond to comparison between the 'Positive cases' and 'Negative cases' columns. Column headings are in pink for Antananarivo and in green for Cambodia

Hematological exam results	Antananarivo Positive cases	Antananarivo Negative cases	Antananarivo Total	Antananarivo p-value	Cambodia Positive cases	Cambodia Negative cases	Cambodia Total	p value
N	10	24	34		35	79	114	
Lymphocytes (/mm³)				0.90				**<0.01**
Median [IQR]	5175 [2024-9900]	4425 [3271-6220]	4425 [3230-7090]		9450 [5600-15500]	6120 [4960-7810]	6505 [5240-9450]	
>9000	3 (30.0)	2 (8.3)	5 (14.7)	0.14	18 (51.4)	13 (16.5)	31 (27.2)	**<0.01**
N	8	22	30		35	80	115	
Leucocytes (/mm³)				0.64				**<0.01**
Median [IQR]	12700 [6985-20950]	15900 [8500-23000]	15750 [8500-23000]		16500 [11000-27300]	11550 [9650-16000]	12400 [10000-18200]	
Neutrophiles (/mm3)	10	24	34		35	78	113	
Neutrophiles (/mm³)				0.39				0.15
Median [IQR]	3450 [2190-6600]	5549 [2380-9385]	4617 [2300-8640]		4680 [3060-8930]	3850 [2840-6170]	3980 [2960-6820]	
N	11	28	39		35	81	116	0.09
Platelets(x1000/mm³)				0.72				
Median [IQR]	360 [139-419]	365 [134-461.8]	360 [139-457]		492 [384-615]	426 [338-521]	446 [367-543]	

a cough for more than 14 days. Forty-seven (22.7%), 56 (27.1%) and 40 (19.3%) infants had a low BMI, height-for-age (stunting) and weight-for-height (wasting), respectively. As described in Supplementary Table 2, apnea, cyanosis, inspiratory whoop and post-tussive vomiting were observed for 39 (18.8%), 86 (41.5%), 92 (44.4%) and 139 (67.1%) patients, respectively. Fever was noted for 34 (16.4%) patients and median [IQR] oxygen saturation was 97% [95–98]. An abnormal pulmonary auscultation was observed for 126 (60.9%) infants (Supplementary Table 2). Table 2 describes reports of pertussis-related symptoms, when data are considered both before and at inclusion.

Biological analyses were performed for 114 infants (55.1%). Among them, 31 (27.2%) infants had more than 9000 lymphocytes/mm^3, with a median [IQR] of 6505 (5240–9450) ranging from 1210 to 34190 lymphocytes/mm^3 (Table 3).

Laboratory diagnosis

Bp and/or *Bpp* PCR diagnosis was provided for 207 infants. A total of 54/207 (26.1%) infants were confirmed as positive cases. There were 51 (24.6%) *Bp* positive cases only, one (0.5%) *Bp/Bpp* coinfection and two (1.0%) *Bpp* positive cases only. No *B. holmesii* positive case was detected.

The characteristics of positive and negative cases are presented in Tables 1 and 2.

Among the 54 positive cases, 45 (83.3%) were under 3 months of age and none had received three vaccine injections. Antibiotic therapy was received by 33 (61.1%) positive cases, of whom four received macrolides, all less than three days before inclusion. Duration of cough at inclusion was more than 7, 10 and 14 days for 40 (74.1%), 25 (46.3%) and 11 (20.4%) positive cases, respectively (Table 1). Apnea, cyanosis, coughing spells and post-tussive vomiting were declared or observed before or at inclusion for 48 (23.2%), 95 (45.9%), 131 (63.3%) and 154 (74.4%) of the positive cases, respectively (Table 2). Pulmonary auscultation, assessed at inclusion, was abnormal for 26 (48.1%) infants (Supplementary Table 2).

As reported in the Table 1, among negative cases, 96 (62.7%) were under 3 months. Antibiotic therapy was given to 87 (56.9%), of whom 15 (17.2%) received

macrolides. Among these fifteen infants, two cases had been exposed to macrolides more than three days before inclusion (one with erythromycin and one with azithromycin).

Regarding hematological analysis, the level of lymphocytes tended to be higher among positive pertussis cases, with 18 infants (51.4%) having more than 9000 lymphocytes/mm^3 relative to 13 (16.5%) infants among the negative cases ($p < 0.01$) (Table 3).

In the multivariate logistic regression analysis (Table 4), several factors were significantly associated with a higher risk of pertussis infection (Table 4): having received less than two doses of vaccine, coughing for more than ten days, coughing spells reported at or before inclusion, presenting with apnea reported at or before inclusion, presenting with normal pulmonary auscultation as well as having someone coughing around the baby.

Clinical status during the final evaluation

The final evaluation was performed significantly earlier among negative cases ($p < 0.001$), at a median timepoint of 7 days [IQR: 6–9] ranging from 1 to 59 days, relative to positive cases, at a median timepoint of 14 days [IQR: 13 to 16] ranging from 3 to 27 days. Of note, 26 (48.1%) positive cases and one (0.7%) negative case presented with persistent cough. Due to the difference in duration of follow-up, we could not compare the two groups. One infant was lost to follow-up (0.5%). Two infants, both positive cases, died (1.0%): one on the day of inclusion and one a day later. The two infants were girls, aged 30 and 75 days, who died after 10 and 11 days of cough, respectively. One girl had had three consultations before inclusion, the other none. Neither had received macrolides. Both presented with coughing spells, apnea, cyanosis, inspiratory whooping, post-tussive vomiting and abnormal pulmonary auscultation.

Hospital-based prospective study in Antananarivo (Madagascar)

Study population

A total of 202 infants were enrolled over a period of 27 months. *Bp* and *Bpp* diagnosis could be evaluated for 173 of them (Fig. 1). Twenty-six patients were excluded due to protocol deviation or high Ct value (> 35).

Among the 173 participants, the gender ratio (M/F) was 1.28. The median [IQR] age was 2.1 [1.4–2.9] months, ranging from 14 days to 6.0 months. The population description is shown in Table 1. Briefly, 133 (76.9%) infants received fewer than two vaccine injections against pertussis. 84 of the 132 infants (63.6%) above 6 weeks of age were compliant with EPI.

Birth weights were low for 34 (19.7%) infants. As reported in Table 1, before inclusion 127 (73.4%) infants had previously attended at least one medical consultation. Antibiotic exposure was reported by 139 (80.3%), of whom 29 (20.9%) independently of any consultation declared. Sixty-one of the 139 patients with antibiotic therapy (43.9%) had received macrolides. As reported in the Supplementary Table 1, apnea, cyanosis, inspiratory whoop and post-tussive vomiting were observed before inclusion for 9 (5.2%), 45 (26.0%) and 27 (15.6%) and 52 (30.1%) infants, respectively.

Regarding the infants' household characteristics, the median [IQR] number of people per household was 5 [4–6] ranging from 2 to 13, including 2 [2–3] adults ranging from 1 to 9, 0 [0–1] between 6 and 15 years old ranging from 0 to 4 children, and 1 [1–2] child between 0 and 5 years old ranging from 0 to 3. Cough in the entourage was reported for 84 (48.6%) individuals.

As reported in the Table 1, at inclusion the median [IQR] duration of cough was 7 [6–11] days, ranging from 5 to 55. Twenty-eight infants (16.2%) presented with more than 14 days of cough. Forty-four (25.4%), 70 (40.5%) and 28 (16.2%) infants had a low BMI, stunting and wasting, respectively. Apnea, cyanosis, inspiratory whoop and post-tussive vomiting were observed for 20 (11.6%), 51 (29.5%), 19 (11.0%) and 33 (19.1%) patients, respectively. Fever was noted for 21 (12.1%) patients. An abnormal pulmonary auscultation was observed for 155 (89.6%) infants. Only eight infants had oxygen saturation recorded. The median [IQR] oxygen saturation was 90.5% [80–95] (Supplementary Table 2).

Biological analyses were performed for 34 infants (19.7%). Among them, 5 (14.7%) had more than 9000 lymphocytes/mm^3, with a median [IQR] of 4425 [3230–7090] ranging from 582 to 16000 lymphocytes/mm^3 (Table 3).

Laboratory diagnosis

A total of 38/173 (22.0%) infants were confirmed as positive cases. There were 26 (15.0%) *Bp* positive cases only, two (1.2%) *Bp/Bpp* coinfections and 10 (5.8%) *Bpp* positive cases only. No *B. holmesii* positive case was detected. The characteristics of positive and negative cases, aggregated across observations preinclusion and at inclusion, are presented in Table 2.

Among the 38 positive cases, one (2.6%) had received three vaccine injections. This infant was a 41-week-old full-term girl with 16 days of cough at inclusion who received her vaccine doses at 6, 11 and 15 weeks. Antibiotic therapy was given to 31 (81.6%) positive cases, of whom 17 (54.8%) received macrolides. Among them, four (23.5%) had received macrolides (erythromycin) more than three days before inclusion. Duration of cough was more than 7, 10 and 14 days for 30 (78.9%), 22 (57.9%) and 13 (34.2%) positive cases, respectively.

Table 4 Factors associated with positivity in Cambodia, based on logistic regression

	Total n=207	Positive cases n=54	OR	[CI 95%] (p)	ORa	[CI 95%] (p)
Period of inclusion						
First semester of 2017 to the end of the first semester of 2018	78 (37.7)	21 (38.9)	ref	--		
Second semester of 2018 to the second semester of 2019	129 (62.3)	33 (61.1)	0.9	[0.5-1.8](0.83)	NS	--
Age						
< 3 months	141 (68.1)	45 (83.3)	3.0	**[1.4-6.5](0.007)**	NS	--
> 3 months	66 (31.9)	9 (16.7)	ref			
Sex						
Boys	110 (53.1)	24 (44.4)	ref			
Girls	97 (46.9)	30 (55.6)	1.6	[0.9-3](0.138)	NS	--
Number of vaccine doses						
0-1 vaccine	143 (69.1)	50 (92.6)	5.5	**[1.9-16.3](0.002)**	7.1	**[2.2-23.1](0.001)**
2-3 vaccine	45 (21.7)	4 (7.4)	ref		ref	
Declarative & missing values	19 (9.2)	0 (0.0)				
Number of consultations before inclusion						
0	39 (18.8)	11 (20.4)	ref			
1	102 (49.3)	18 (33.3)	0.5	[0.2-1.3](0.169)	NS	--
2 ore more	66 (31.9)	25 (46.3)	1.6	[0.7-3.7](0.314)		
Cough longer than 10 days						
No	146 (70.5)	29 (53.7)	ref		ref	
Yes	61 (29.5)	25 (46.3)	2.8	**[1.5-5.4](0.002)**	2.6	**[1.2-5.7](0.017)**
Low BMI for age						
No	160 (77.3)	46 (85.2)	ref			
Yes	47 (22.7)	8 (14.8)	0.5	[0.2-1.2](0.112)	NS	--
Number of adults in the family						
1-3 persons	114 (55.1)	28 (51.9)	1.2	[0.6-2.2](0.58)	NS	--
>= 4 persons	93 (44.9)	26 (48.1)	ref			
Number of children younger than 6 years old in the family						
0-1 children	111 (53.6)	29 (53.7)	ref			
>=2 children	96 (46.4)	25 (46.3)	1.0	[0.5-1.9](0.989)	NS	--
Number of children between 6 and 15 years old in the family						
0 children	111 (53.6)	22 (40.7)	ref			
>= 1 children	96 (46.4)	32 (59.3)	2.0	**[1.1-3.8](0.029)**	NS	--
Someone coughing and in close contact with the infant						
No	80 (38.6)	12 (22.2)	ref		ref	
Yes	127 (61.4)	42 (77.8)	2.8	**[1.4-5.7](0.005)**	3.1	**[1.4-7.1](0.007)**
Good conditions btw cough at inclusion						
No	44 (21.3)	8 (14.8)	ref			
Yes	162 (78.3)	46 (85.2)	1.8	[0.8-4.1](0.176)	NS	--
Missing values	1 (0.5)	0 (0.0)	--			
Pulmonary auscultation						
Normal	81 (39.1)	28 (51.9)	2.0	**[1.1-3.8](0.027)**	2.7	**[1.3-5.9](0.011)**
Abnormal	126 (60.9)	26 (48.1)	ref		ref	
Fever						
No	173 (83.6)	49 (90.7)	2.3	[0.8-6.3](0.106)	NS	--
Yes	34 (16.4)	5 (9.3)	ref			
Coughing spells at and before inclusion						
No	76 (36.7)	11 (20.4)	ref		ref	
Yes	131 (63.3)	43 (79.6)	2.9	**[1.4-6](0.005)**	3.3	**[1.4-7.7](0.005)**
Apnea at and before inclusion						
No	159 (76.8)	31 (57.4)	ref		ref	
Yes	48 (23.2)	23 (42.6)	3.8	**[1.9-7.6](< 0.001)**	3.6	**[1.6-8.3](0.002)**
Cyanosis at and before inclusion						
No	112 (54.1)	21 (38.9)	ref			
Yes	95 (45.9)	33 (61.1)	2.3	**[1.2-4.4](0.01)**	NS	--
Inspiratory whoop at and before inclusion						
No	104 (50.2)	23 (42.6)	ref			
Yes	102 (49.3)	31 (57.4)	1.5	[0.8-2.9](0.178)	NS	--
Missing values	1 (0.5)	0 (0.0)	--			

Table 5 Factors associated with positivity in Madagascar, based on logistic regression

	Total n=173	Positive cases n=38	OR	[CI 95%] (p)	ORa	[CI 95%] (p)
Period of inclusion						
First semester of 2017 to the end of the first semester of 2018	95 (54.9)	16 (42.1)	ref			
Second semester of 2018 to the second semester of 2019	78 (45.1)	22 (57.9)	1.9	[0.9-4.0](0.075)	NS	--
Number of consultations before inclusion						
0 consultation	46 (26.6)	6 (15.8)	ref			
1 consultation	66 (38.2)	10 (26.3)	1.2	[0.4-3.5](0.754)		
2 or more consultation	60 (34.7)	21 (55.3)	3.6	**[1.3-9.8](0.013)**	NS	--
Missing values	1 (0.6)	1 (2.6)				
Cough longer than 10 days						
No	116 (67.1)	16 (42.1)	ref		ref	
Yes	57 (32.9)	22 (57.9)	3.9	**[1.9-8.3](<0.001)**	3.6	**[1.7-7.6](0.001)**
Low weight-for-height						
No	113 (65.3)	27 (71.1)	ref			
Yes	28 (16.2)	3 (7.9)	0.4	[0.1-1.4](0.139)	NS	--
Missing values	32 (18.5)	8 (21.1)				
Number of adults in the family						
1-3 persons	132 (76.3)	26 (68.4)	Ref			
>= 4 persons	41 (23.7)	12 (31.6)	1.7	[0.8-3.7](0.199)	NS	--
Number of children younger than 6 years old in the family						
0-1 children	90 (52.3)	23 (62.2)	1.7	[0.8-3.5](0.179)	NS	--
>=2 children	82 (47.7)	14 (37.8)	ref			
Number of children between 6 and 15 years old in the family						
0 children	96 (55.5)	21 (55.3)	ref			
>= 1 children	77 (44.5)	17 (44.7)	1	[0.5-2.1](0.974)	NS	--
Someone coughing and in close contact with the infant						
No	89 (51.4)	22 (57.9)	ref			
Yes	84 (48.6)	16 (42.1)	0.7	[0.3-1.5](0.369)	NS	--
Pulmonary auscultation						
Normal	18 (10.4)	7 (18.4)	2.5	[0.9-7.1](0.074)	NS	--
Abnormal	155 (89.6)	31 (81.6)	ref			
Post-tussive vomiting at and before inclusion						
No	109 (63.0)	20 (52.6)	ref			
Yes	64 (37.0)	18 (47.4)	1.7	[0.8-3.6](0.136)	NS	--
Declaration of good condition between cough, before inclusion						
No	113 (65.3)	18 (47.4)	ref			
Yes	60 (34.7)	20 (52.6)	**2.6**	**[1.3-5.5](0.01)**	2.3	**[1.1-4.9](0.036)**
Declaration of nocturnal cough, before inclusion						
No	57 (32.9)	8 (21.1)	ref			
Yes	116 (67.1)	30 (78.9)	2.1	[0.9-5](0.082)	NS	--

Apnea, cyanosis, coughing spells and post-tussive vomiting were declared or observed before or at inclusion for 5 (13.2%), 15 (39.5%), 34 (89.5%) and 18 (47.4%) of the positive cases, respectively. Pulmonary auscultation, assessed at inclusion, was abnormal for 31 (81.6%) infants.

As reported in Table 1, among negative cases, 104 (77.0%) were under three months. Antibiotic exposure was reported in 108 (80.0%) children, of whom 44 (40.7%) had received macrolides. Duration of macrolide exposure was above three days before inclusion for fourteen of them (31.8%), specifically erythromycin for twelve, pristinamycin and spiramycin for the two others. Five of these fourteen infants (35.7%) had had no previous consultation.

In the hematological analysis, there was no difference regarding the level of lymphocytes between the positive and negative pertussis cases ($p=0.9$) (Table 3).

In the multivariate logistic regression analysis, several factors were significantly associated with a higher risk of pertussis infection (Table 5): coughing for more than ten days and presenting clinically well between coughing spells, reported before inclusion.

Clinical status during the final evaluation

The final evaluation was performed significantly earlier among negative cases ($p<0.001$), at a median timepoint of 4 days [IQR: 3–6] ranging from one to 27 days, relative to positive cases, at a median timepoint of 13 days [IQR: 13 to 14] ranging from 1 to 17 days. Because of this

difference, final status could not be compared between the infected and noninfected infants. Five negative cases were lost to follow-up (3.7%) and three infants, all positive cases, died (1.7%) during the course of the study. These infants were one girl and two boys, aged 43, 38 and 56 days, who died two, three and seven days after inclusion, respectively. They had been coughing for less than seven days. Before inclusion, the girl had attended two consultations, one boy had not attended any and the other boy had attended one. The girl had received erythromycin one day before inclusion and had low BMI, and one of the two boys also had low BMI compared to the second.

Comparison between populations studied in Cambodia and Madagascar

The infants in the two groups presented with a similar duration of cough at inclusion (median [IQR], 7 [6–11] in Madagascar, 7 [6–10] in Cambodia, p-value > 0.3), and with a similar positivity rate (22.0% in Madagascar, 26.1% in Cambodia, p-value > 0.3). In Antananarivo, infants were significantly younger ($p < 0.02$), with a higher proportion of premature births ($p < 0.001$), low birth weights ($p < 0.001$) and stunting ($p < 0.001$). Of note, abnormal pulmonary auscultation was more often reported in the Madagascan infants when the general study population was considered ($p < 0.001$), as well as when positive cases were considered ($p < 0.001$).

Contact-case investigation in Cambodia

During the contact-case investigation, households of the 54 positive index cases were investigated. A total of 232 individuals were recruited; all accepted the NPS and blood sampling (Fig. 1). The median [IQR] age of contact cases was 26.7 [9.3–36.9] years, ranging from 1.1 to 79.7 (Supplementary Table 3). Among them, 151 (65.1%) were females and 151 (65.1%), 48 (20.7%) and 33 (14.2%) were adults (i.e. ≥15 years old), children aged 6 to 15 and children under 5, respectively. The median [IQR] number of contact cases investigated per household was 5 [4–5], ranging from 2 to 5. Contact cases consisted of 50 (21.6%) mothers; 43 (18.5%) grandparents; 42 (18.1%) siblings and cousins above 5 years old; 35 (15.1%) uncles or aunts; 32 (13.8%) fathers; and 30 (12.9%) siblings and cousins 5 years old or younger (Table Supplementary 3).

Two thirds of the contact cases (178 (76.7%)) presented with cough.

Laboratory diagnosis

The median [IQR] interval between index identification as a positive case and contact investigation was 8 [6–9] days and ranged from 3 to 13. The median [IQR] interval between the onset of symptoms in index cases and the investigation was 16 [12–19] days, ranging from 9 to 32.

Bp and/or *Bpp* positive cases were confirmed by PCR and/or serology for 118/232 (50.9%) contact cases, among which 75/232 (32.3%) by PCR and 74/232 by serology (31.9%), with a serology titration above the 40 IU/mL threshold. Both positive serology and PCRs were observed for 31 (13.4%) contact cases. In the PCR results, *Bp*, *Bpp* cases and coinfections were found for 70 (30.2%), 2 (0.9%) and 3 (1.3%) individuals, respectively. *B. holmesii* were detected for two individuals, with no coinfection. 36/232 (15.5%) had a serology result above 100 UI/mL.

The median [IQR] number of positive cases by PCR or serology investigated per household was 2 [1–3], ranging from 0 to 5, with a median of 50% [30–80] of positive cases in a household, ranging from 0–100%. A large majority of households (51/54; 94.4%) had at least one positive case. If only *Bp*-positive index households are considered (infection or coinfection), the proportion of households with at least one case positive by PCR or serology was 96.2% (50/52).

No pertussis infection was detected in three households, representing twelve people. The index cases from these three households consisted of two *Bp* cases and one *Bpp* case. For one *Bp* case, four adults were recruited for the CCI. For the other *Bp* case, two adults and one child were recruited. A vast majority of these individuals reported cough (83.3%, 10/12), with a median [IQR] duration of 8 [3–11] days.

Contact-case investigation in Antananarivo (Madagascar)

During the contact-case investigation, 32 households were included with 128 individuals. Six households were not investigated due to unwillingness to participate. PCR and serology analyses were carried out on 124 participants following the exclusion of four participants belonging to the same household. Two more participants were excluded from PCR analyses due to a Ct value above 35, and one due to an aberrant PCR result, resulting in 121 individuals with PCR results. One individual was excluded from the serology analysis due to a problem with the diagnosis by ELISA, resulting in 120 individuals with ELISA results. The individuals belonged to 31 households (Fig. 1).

The median [IQR] age of contact cases was 19.9 [7.45–30.25] years, ranging from 1.6 to 68.5 years (Supplementary Table 3). Among them, 71 (58.7%) were females and 71 (58.7%), 28 (23.1%) and 22 (18.2%) were adults (i.e. older than 15 years), children aged 6 to 15 and children 5 years old or below, respectively.

The median [IQR] number of contact cases investigated per household was 4 [3–5], ranging from 1 to 6 individuals. Contact cases consisted of 29 (24.0%) mothers; 22

(18.2%) siblings and cousins under 5 years; 22 (18.2%) siblings and cousins above 5 years; 19 (15.7%) fathers; 16 (13.2%) uncles or aunts; 11 (9.1%) grandparents and two (1.7%) nurses (Table Supplementary 3).

Just over half of the contact cases (63 (53.7%)) presented with coughs.

Laboratory diagnosis

The median [IQR] interval between index identification as a positive case and contact investigation was 7 [4–8] days and ranged from 3 to 13. The median [IQR] interval between the onset of symptoms in index cases and the contact case investigation was 18 [14–21] days, ranging from 7 to 44. *Bp* and/or *Bpp* positive cases were confirmed by PCR and/or serology for 82/121 (67.8%) contact cases, among which 71/121 (58.7%) by PCR and 35/121 by serology (28.9%). PCR results revealed that *Bp*, *Bpp* infection and coinfection were found for 58 (47.9%), 7 (5.8%) and 6 (5.0%) individuals, respectively. No *B. holmesii* positive case was detected.

The median [IQR] number of positive cases by PCR or serology per household was 3 [2–4], ranging from 0 to 6, with a median of 80% [40–100] of positive cases among all the contacts in a household, ranging from 0–100%. A large majority of households (28/31, 90.3%) had at least one positive case. When only *Bp*-positive index households were considered (infection or coinfection), the proportion of households with at least one case positive by PCR or serology was 96.0% (24/25). No pertussis infection was detected in three households, representing nine people. The index cases of these households were two *Bpp* cases and one *Bp* case. For the Bp case, only two adults were recruited during the CCI. Three out of nine (33.3%) individuals reported cough, with a median [IQR] duration of five [2–7] days; none reported apnea, cyanosis or vomiting. Inspiratory whoop was reported by one of them (11.1%).

Discussion

Using PCR and serology, we evidenced high pertussis circulation in Cambodia and Antananarivo among infants and their contacts across all age categories.

Bp or *Bpp* positive cases were confirmed in 26.1% and 22.0% of the infants in Cambodia and Antananarivo, respectively.

In Cambodia, 51 (24.6%) infections were caused by *Bp* versus 26 (15.0%) in Antananarivo. Two infants in Cambodia (1.0%) and ten in Antananarivo (5.8%) were *Bpp* positive cases. In Cambodia and Antananarivo, one infant (0.5%) and three infants (1.2%) respectively were coinfected with *Bp* and *Bpp*. This study therefore shows that pertussis circulates highly in both Cambodia and Antananarivo, with noticeably more *Bpp* infections in Antananarivo than in Cambodia, as well as compared to Tehran data collected with a similar protocol [11].

It is worth noting that the high number of infections reported here might be underestimated, especially in Antananarivo, where two Madagascan infants with a negative PCR had been coughing for 45 and 50 days at inclusion. Moreover, fourteen (8.0%) of the negative cases had received macrolides more than three days before inclusion. In Cambodia, two infants were affected (9.0%). Generally, the study reveals high exposure to antibiotics, especially in Antananarivo, where over four in five infants (80.3%) had received antibiotic therapy, versus 58.0% in Cambodia, raising questions over the possible impact of prescription on antibiotic resistance in these two geographical areas. Our study also revealed antibiotic uptake independently of medical consultations for as much as 20.9% of the cohort in Antananarivo (resp. 10.8% in Cambodia). These antibiotics were presumably bought at traditional or urban markets and have unknown effectiveness and toxicity. It is important to note that in Antananarivo, four positive cases had received macrolides (erythromycin) more than three days before inclusion. Our data do not enable us to decipher whether this is linked to a problem with absorbance, the fact that the macrolides could not be fully ingested for various reasons relating, for instance, to the population's young age, or to pertussis strains resistant to macrolides.

Regarding deaths, all died within the first week of inclusion. Duration of cough and number of consultations before inclusion among these children highlight both the disease's potential severity and the need for early diagnosis.

The multivariate analysis shows that coughing for more than ten days at inclusion is a risk factor for pertussis in both the Madagascan and Cambodian cohorts, consistent with the known long duration of cough in pertussis cases [16, 17]. In addition, consistent with a pertussis protection level of at least 80% with two doses [18], having received at least two vaccine doses appears to be a protective factor in Cambodia. In this cohort, infants under three months were overrepresented among the sick infants, highlighting the vulnerability of this population, too young to receive two or three doses of vaccine. Indeed, virtually all deaths by pertussis occur, in the present era, in infants under four months [16], and the vulnerability of infants under three months has repeatedly been shown, recently in Mexico [19] and Panama [20] for instance. More surprisingly, having received two doses does not appear to be a protective factor in Madagascar, although older infants had received more doses than younger infants, as expected. Several factors might explain this result, such as weak effectiveness of the vaccine itself, which varies greatly across wPV producers [6].

Issues with cold chain management have been reported as an area of improvement in African countries and could also be at play [21]. Finally, it is possible that nutritional deficiencies weakened the vaccines' protective effect in some infants since the literature suggests links between malnutrition and ineffective immunological memory mechanisms [22, 23]. In Madagascar, nearly 40% of children are malnourished, and 40.5% of our cohort had stunting, a possible sign of malnutrition.

Clinical signs identified as pertussis risk factors in Cambodia were the following: presenting with normal pulmonary auscultation, coughing spells or apnea. The presence of someone coughing and in close contact also appeared, unsurprisingly, to be a risk factor. These findings are consistent with the symptomatology of pertussis commonly described in infants. Greenberg *et al.* report that coughing spells, when they are observed, occur more than twelve times per day and at night in this population, and that 35–53% of neonates present with apnea [17], while normal chest examination has been described as a typical sign for pertussis [16]. The results of hematological analyses were also consistent with the literature [16, 17], with more hyperlymphocytosis and hyperleukocytosis observed among positive than negative cases.

In contrast with the multitude of risk factors identified in the Cambodian cohort, in Antananarivo, apart from cough longer than ten days, the only risk factor identified was being clinically well between coughing spells, a characteristic also found in the literature [17]. An important observation though is that 89.6% of the Madagascan cohort presented with abnormal pulmonary auscultation, significantly more than in Cambodia (60.9%), suggesting a very high rate of bacterial coinfections. In Madagascar, acute respiratory infections from etiologies other than pertussis are frequent [24, 25] and the situation was possibly worsened here by a plague outbreak from August 2017 to November 2017, overlapping with the recruitment period. In addition, the infants might have been more likely to develop respiratory tract infections due to nutritional deficiencies [26, 27].

On one hand, the long list of risk factors identified in Cambodia validates the robustness of the study. On the other, coinfections with pertussis and additional bacteria possibly obscured clinical presentation of the disease in Antananarivo, leading to only two identified risk factors. This is a general reminder of the critical importance of laboratory techniques such as PCR and serology to detect the disease reliably due to the lack of specific symptoms in cases of whooping cough [28] and especially in infants, who appear deceptively well [16].

With 353 contact cases investigated in total with PCR and serology, we present here the results of a large investigation study. The CCI shows the high circulation of pertussis among individuals in all age categories and very high transmission within households, thereby extending the results obtained in infants. More than two thirds (67.8%) of the contact cases were infected in Antananarivo, with positive cases found in 90.3% of the households. Over half of the contact cases were infected in Cambodia (50.9%), with positive cases found in 94.4% of the households. Ten percent of the contact cases were found to be *Bpp* positive cases or *Bp*/*Bpp* cases in Antananarivo, five times more than in Cambodia. The composition of investigated households and the lack of serological detection of *Bpp* infection may explain the rare cases of households with no positive cases detected.

The evidence we find of substantial circulation in the households of infected infants is consistent with the few but recent seroepidemiological studies conducted in these two countries [8, 9, 29].

Limitations

As discussed previously, previous exposure to macrolides and/or long duration of cough could lead to an underestimation of the number of positive cases. Indeed, PCR positivity starts to decrease after 21 days of cough [30, 31]. Another limitation of the study is that, in Antananarivo, we had a very high number of missing values in the hematological results, certainly due to lack of access to laboratory tests for financial reasons. For the same reasons, we have no information on co-infections with pertussis. As for the CCI, we might have missed positive contact cases infected with *Bpp*, and therefore slightly underestimated the level of infection in households, since serology cannot detect *Bpp*.

Conclusion

We have highlighted high pertussis circulation among infants with at least five days of cough and at least one pertussis-like symptom, as well as in their households, across all age categories. Given the vulnerability of young infants, who are too young to receive full primary vaccination and of whom five died in this study, boosters are clearly needed to interrupt transmission chains. Madagascar's results further indicate that laboratory access to dedicated molecular diagnostics is key to detect cases early independently of symptomatology, and act effectively at an individual and collective level. This is especially needed in settings where the clinical presentation can be obscured by secondary infections.

Abbreviations

Anti-PT IgG	Pertussis toxin immunoglobulin G
aPV	acellular vaccines
Bp	Bordetella pertussis
Bpp	Bordetella parapertussis
CCI	contact-case investigation
ELISA	enzyme-linked immunosorbent assay

EPI	Expanded Programme on Immunization
HIC	high income countries
IPC	Institut Pasteur of Cambodia
IPM	Institut Pasteur of Madagascar
LLOQ	lower limit of quantitation
LMIC	low- and middle- income countries
NISBC	National Institute for Biological Standards and Control
NPS	nasopharyngeal swab
PS	prospective study
qPCR	quantitative polymerase chain reaction
wPV	whole-cell vaccines
WHO	World Health Organization

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12879-025-10590-6.

Additional File 1. Supplementary Table 1. Symptomatology of the population, before inclusion in the PS. Columns headings are in pink for Antananarivo and in green for Cambodia. *Patients with post-tussive vomiting are considered as having vomiting as well.

Additional File 2. Supplementary Table 2. Symptomatology of the population, at inclusion in the PS. Columns headings are in pink for Antananarivo and in green for Cambodia.

Additional File 3. Supplementary Table 3 Population in the contact-case investigation. Columns headings are in pink for Antananarivo and in green for Cambodia. * One individual had no serology data.

Additional File 4. Supplementary Figure 1. Biological exam results.

Additional File 5.

Additional File 6.

Additional File 7.

Additional File 8.

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†**PERILIC working group**:
Annick ROBINSON[a], Mbolanirina L RAKOTOMAHEFA NARISON[b]Moniboth DUCH[c], Peuv CHEA[d], Vantha TE[e], Sokha POL[e], Ka TENG[f], Kimsroin KIM[g], Thona SAR[h], Tek Chheng EAP[i], Kong SOK[g], Hortensia M. Heriniaina [j], Mializay Ralalanombana [j], Solohery L. Razafimahatratra [j], Chanthy Keang [k], Kimhab Ky[k]

Affiliations
[a]Department of Child Health, Teaching Hospital, Centre Hospitalier Universitaire Mère Enfant Tsaralalana, Antananarivo, Madagascar
[b]Joseph Ravoahangy Andrianavalona Hospital, Antananarivo Madagascar
[c]National Pediatric Hospital, Phnom Penh, Phnom Penh, Cambodia
[d]Battambang Provincial Hospital, Battambang, Cambodia
[e]Takeo Provincial Hospital, Takeo, Cambodia Cambodia
[f]Provincial Hospital, Kampot, Cambodia
[g]Provincial Hospital, Kompong Cham, Cambodia
[h]Sonja Kill Memorial Hospital, Kampot, Cambodia
[i]Apsara Phnom Penh Clinic, Phnom Penh, Cambodia
[j]Bacteriology Experimental Unit, Institut Pasteur de Madagascar, Antananarivo, Madagascar
[k]Epidemiology and Public Health Unit, Institut Pasteur du Cambodge, Phnom Penh, Cambodia

Contact
Annick ROBINSON annicklalaina@yahoo.fr
Mbolanirina L RAKOTOMAHEFA NARISON mahefambola@yahoo.fr
Moniboth DUCH, monibothduch@yahoo.com
Peuv CHEA, cheapeuv@gmail.com
Vantha TE , pediatrh@yahoo.com
Sokha POL, polsokha66@gmail.com
Ka TENG
Kimsroin KIM, kimkimsrorn@yahoo.com
Thona SAR, sarthona@yahoo.com
Tek Chheng EAP, techheng@yahoo.com
Kong SOK, sokkong555@gmail.com
Hortensia M. Heriniaina, hortensia@pasteur.mg
Mializay Ralalanombana, mializay@pasteur.mg
Solohery L. Razafimahatratra, solohery@pasteur.mg
Chanthy Keang, kchanthy@pasteur-kh.org
Kimhab Ky, kkimhab@pasteur-kh.org

Authors' contributions
GN: Data Curation; Formal Analysis; Data Interpretation; Writing. AH; Investigation; Review and Editing. LB: Investigation; Supervision; Review and Editing. FC: Data Interpretation; Writing of the original manuscript. CL: Investigation. CB: Laboratory Analysis. LR: Laboratory Analysis; Data Curation; Review and Editing. MR: Data Curation; Formal analysis. AK: Laboratory Analysis; Supervision. BD: Investigation. AMR: Data Curation. MAA: Study Design. NG: Study Design; Methodology; Supervision; Formal Analysis; Data Interpretation; Writing and Editing; JMC: Supervision; Review and Editing. FT: Study Design; Methodology; Supervision; Formal Analysis; Data Interpretation; Writing and Editing.

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Data availability
The datasets used and analysed during the current study are available from the corresponding author on reasonable request, via Zenodo, with the https://doi.org/10.5281/zenodo.13734735.

Declarations

Ethics approval and consent to participate
The study was conducted under the sponsorship of the Institut Pasteur, Paris. The protocol was reviewed and approved by the IP's Institutional Review Board, the National Biomedical Research Ethics Committee in Madagascar (N° 065-MSANP/CE), and the National Ethics Committee for Health Research in Cambodia (N° 019NECHR). All procedures were in accordance with the World Medical Association's Declaration of Helsinki (2008). Authorization for data processing was obtained from the French Data Protection Authority (CNIL), and names were pseudonymized by assigning a study-specific code to each participant. The ClinicalTrials.gov identifier of the study is: NCT02983487, which was first registered on the 2nd of December 2016.
Informed written consent was obtained from both the adult participants and the parent(s)/legal guardian(s) of all under-18s (minor children). The age under which parent(s)/legal guardian(s) consented for the individuals (i.e. 18 years) was determined by national ethics committee, which followed national regulations. Oral assent was obtained for all the children of age seven years or older. Only individuals who agreed to participate were included in the study.

Consent for publication
Not Applicable.

Competing interests
The authors declare no competing interests.

Author details
[1]Center for Translational Science, Institut Pasteur, Paris cité University, Paris, France. [2]Epidemiology and Clinical Research Unit, Institut Pasteur de Madagascar, Antananarivo, Madagascar. [3]Epidemiology and Public Health Unit, Institut Pasteur du Cambodge, Phnom Penh, Cambodia. [4]Institut Pasteur Medical Center, Paris-cité University, Paris, France. [5]Medical Biology Unit, Institut

Pasteur du Cambodge, Phnom Penh, Cambodia. [6]Bacteriology Experimental Unit, Institut Pasteur de Madagascar, Antananarivo, Madagascar. [7]Centre for Translational Science, Clinical Coordination, Institut Pasteur, Université de Paris, Paris, France.

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