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In vitro activity and clinical efficacy of macrolides, cefoperazone-sulbactam and piperacillin/piperacillin-tazobactam against *Bordetella pertussis* and the clinical manifestations in pertussis patients due to these isolates: A single-centre study in Zhejiang Province, China

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ABSTRACT

Background and objective: Macrolides are the recommended antibiotics for treating pertussis and preventing transmission. The causative bacterium, *Bordetella pertussis*, has high macrolide resistance and has recently circulated in China. The objective of this study was to find effective alternative antibiotics for treatment by assessing the in vitro activity and clinical efficacy of antibiotics against *Bordetella pertussis*.
Methods: Bordetella pertussis was confirmed by agglutination with specific antisera and mass spectrometry. The MICs of antibiotics against isolates were determined using the Etest method. Treatment outcomes were clinically and microbiologically evaluated.
Results: A total of 126 pertussis patients were diagnosed based on culture, 69.8% of whom were aged ≤6 months and 72.1% were treated with previous macrolides. Leucocytosis and lymphocytosis were observed in 29.4% and 54.8% of all patients, respectively. Both MIC_{50} and MIC_{90} of erythromycin, azithromycin, and clindamycin were >256 mg/L, and 75.4% were highly macrolide resistant. The MIC_{90} of trimethoprim-sulfamethoxazole, ampicillin, ampicillin-sulbactam, cefuroxime, ceftriaxone and cefoperazone-sulbactam were 0.38 mg/L, 0.25 mg/L, 0.19 mg/L, 12 mg/L, 0.19 mg/L and 0.047 mg/L, respectively. The MICs of piperacillin in all of the isolations were <0.016 mg/L. Of the patients treated with single cefoperazone-sulbactam or piperacillin-tazobactam, 30 of 32 (93.8%) had significantly improved clinical symptoms and 24 of 25 (96%) had negative culture results after 2 weeks of therapy.
Conclusion: Macrolide resistance in *Bordetella pertussis* is a serious problem in Zhejiang Province, China. Piperacillin/piperacillin-tazobactam and cefoperazone-sulbactam have potent antibacterial activity in vitro and in vivo, and may become the alternative choice for treating pertussis caused by macrolide-resistant isolates.

1. Introduction

Pertussis, also known as whooping cough, is an acute respiratory tract infection caused by *Bordetella pertussis* (*B. pertussis*). Typical pertussis is characterised by severe, long-lasting, spasmodic, paroxysmal coughing after its initial onset in children, and it is particularly serious in neonates and infants [1,2]. In recent years, the number of pertussis cases has significantly increased, and it is re-emerging as a public health problem, even in regions with high vaccine coverage [3–5]. However, antibiotics for treating pertussis are extremely limited, and macrolide antibiotics, such as erythromycin and azithromycin, remain the only recommended antibiotics for the treatment and prophylaxis of this disease [6]. Early intervention with sensitive antibiotics is beneficial and associated with a shortened duration of coughing and reduced risk of death in young infants [1]. Usually, macrolide resistance is not a major concern for pertussis [6,7], although

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erythromycin resistance in *B. pertussis* has been described in some countries [8–10].

Recently, Wang [11] and Yang et al. [12] found that *B. pertussis* isolates with high levels of macrolide resistance are very common in some parts of China; thus, alternative antibiotics to treat paediatric pertussis caused by macrolide-resistant strains, or patients for whom treatment has failed, is urgently needed. This study sought to assess the in vitro activity and clinical efficacy of non-macrolide antibiotics – such as piperacillin/piperacillin-tazobactam and cefoperazone-sulbactam – against *B. pertussis* isolates to find effective alternative antibiotics for treatment.

2. Materials and methods

2.1. Ethics

The study was approved by the Local Research Ethics Committee of the Children's Hospital, Zhejiang University School of Medicine (2016-IRB-014), and informed consent was obtained for experimentation. This research was conducted in accordance with the Declaration of Helsinki and national and institutional standards.

2.2. Patients

The patients with suspected pertussis and enrolled in the study were those with a cough lasting >2 weeks, or had at least one of the symptoms of paroxysmal cough, whoop, or post-tussive vomiting [13]. Nasopharyngeal swabs, one from each patient, were collected upon admission to the Division of Infectious Disease or Respiratory Department in 2016.

2.3. Organism culture

All nasopharyngeal swabs were inoculated onto charcoal agar (CM0119, Oxoid, UK) plates supplemented with 10% defibrinated sheep blood and 40 mg/L of cephalexin. The inoculation was completed within 1 h of specimen collection. Inoculated agar plates were incubated in humid air at 36 °C for 7 days. Small, mercury-drop-like colonies were observed, suspected of being *B. pertussis*, subcultured, and Gram stained. *Bordetella pertussis* strains were confirmed by slide agglutination with specific antisera against *B. pertussis* (Remel Europe Ltd., UK) and a negative reaction with *Bordetella parapertussis* antiserum (Remel Europe Ltd., UK). Further identification was completed by mass spectrometry analysis.

2.4. Antibacterial agents

The following antibiotics were tested: erythromycin (0.016–256 mg/L, lot 1004135240); azithromycin (0.016–256 mg/L, lot 1004325160); clindamycin (0.016–256 mg/L, lot 1004182730); ampicillin (0.016–256 mg/L, lot 1004177470); ampicillin-sulbactam (0.016–256 mg/L, lot 1004810420); cefuroxime (0.016–256 mg/L, lot 1004498100); ceftriaxone (0.002–32 mg/L, lot 1004755930); cefoperazone-sulbactam (0.016–256 mg/L, lot 1003310140); piperacillin (0.016–256 mg/L, lot 1005194760); sulfamethoxazole-trimethoprim (0.002–32 mg/L, lot 1003652350); and meropenem (0.016–256 mg/L, lot 1005206660). All of the above antibiotic strips were produced by bioMériex Ltd (France).

2.5. MIC determination

The MICs of antibiotics were determined using the Etest method. *Bordetella pertussis* strains for testing were subcultured on charcoal agar plates with 10% defibrinated sheep blood (without cephalexin) for 72 h. Then strains were collected with sterile cotton swabs and transferred in sterile glass tubes with 0.45% sodium chloride solution. The organism suspensions were adjusted to 0.5 McFarland standard for Etest, based on the standard recommended by the Clinical and Laboratory Standard Institute (CLSI) M100 S26, and were then daubed on charcoal agar plates (without sheep blood or cephalexin, 25 mL of medium in each 90-mm plate) with sterile cotton swabs. Etest strips of antibiotics were pasted on the plates (maximum of two strips on each plate). Inoculated plates with Etest strips were incubated for 72 h at 36 °C in ambient air, and MICs of antibiotics were determined according to the instructions included with the Etest products. The quality control strains used were *Staphylococcus aureus* ATCC 29213 and *Escherichia coli* ATCC 25922, and their tested MICs were within the acceptable ranges recommended by the CLSI.

2.6. Treatment

Erythromycin (10 mg/kg q8 h, Hunan Kelun, China) or azithromycin (10 mg/kg q24 h, Pfizer, Dalian, China) were prescribed as the initial antibiotics for the patients who had not been given any previous antibiotics, or those treated with macrolides for <7 days [10]. For those whose symptoms did not improve after ≥7 days of macrolide intervention, cefoperazone-sulbactam (60 mg/kg q12 h, Pfizer, Dalian China) or piperacillin-tazobactam (40 mg/kg q8 h, ZheJiang Medicine Co., China; piperacillin was unavailable) was selected. For those whose symptoms improved after treatment with β-lactams at pre-admission, the same previous antibiotics were continued. Treatment outcomes were recorded and patients were discharged from hospital after 14 days of treatment if clinical symptoms significantly improved. Post-treatment cultures were administered weekly in some patients. Patients were followed up for 4 weeks after they were discharged from hospital.

2.7. Statistical analysis

Normal distribution data were presented as mean ± standard deviation ($\bar{x} \pm s$). Comparison of normal distribution data between groups was performed using t test. Abnormal distribution data were presented using the median. Comparison of abnormal distributed data between groups was performed using Mann-Whitney U test. Comparison of enumeration data between groups was performed using Fisher's exact test. $P < 0.05$ was considered to be of statistical significance.

3. Results

3.1. Demographic information and clinical features

A total of 948 patients from Zhejiang Province with suspected pertussis and aged from 21 days to 14 years (median age 5 months and 21 days, 61.8% male) were enrolled between January 1 and December 31 2016. A total of 692 (73.0%) were aged <1 year. Nasopharyngeal swabs were positive for *B. pertussis* and *Bordetella parapertussis* by culture in 126 (13.3%) and four (0.4%) of all patients, respectively. The patients with positive culture results of *B. pertussis* were significantly younger than those with negative culture results (median ages were 3 months 27 days vs. 6 months and 3 days, $Z = 3.60$, $P < 0.001$) and 88 of 126 (69.8%) of the pertussis patients were aged <6 months.

Fig. 1 shows the distribution of pertussis patients with positive culture results in each month in 2016. Of these, 84 of 126 (66.7%) were identified in May, June, July and August. *Bordetella pertussis* strains were isolated when the patients had been coughing for 3–60 days (17.1 ± 10.3). Prior to the initial onset in patients, 82 of 126

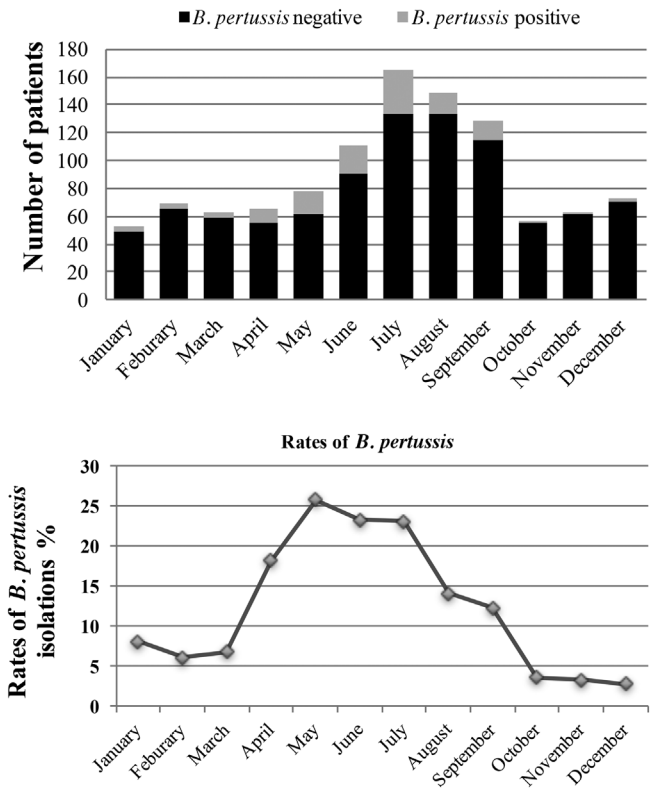

Fig. 1. Distribution of *Bordetella pertussis* patients identified by culture during each month of 2016.
Note: The figure shown above gives the numbers of patients with (126) or without (822) *Bordetella pertussis* as indicated by culture from nasopharyngeal swabs in each month. The figure below shows the rates of isolation of *B. pertussis* from nasopharyngeal swabs during each month, with the highest (25.8%) in May 2016. Six isolates were genetically and epidemiologically related (from three families, three pairs of siblings).

(65.1%) of the mothers, 55 (43.7%) of the fathers, and 57 of 69 (82.6%) of the siblings reported having a persistent cough lasting more than 1 week. *Bordetella pertussis* was isolated in 41 of 76 (53.9%) of the mothers, 19 of 76 (25.0%) of the fathers, and 21 of 43 (48.8%) of the siblings. Of all 126 patients, 76 (60.3%) were unvaccinated, and 21 (16.7%) had been vaccinated with one or two doses of diphtheria-pertussis-tetanus vaccine. Ninety-eight (72.1%) patients had undergone previous macrolide treatments for 1–14 days (5.0 ± 2.9). Ten (7.9%) patients did not receive any antibiotics prior to hospitalisation.

Of all 126 culture-confirmed pertussis patients, 64 (50.8%) had a cough accompanied by bruising, 16 (12.7%) with wheezes, and 17 (13.5%) had short-term fever. Moist rales were heard in 36 (28.6%) cases. Signs of condensation were seen on chest radiographs in 47 (37.3%) of the patients, including two with localised emphysema, one with localised atelectasis, and one with a pleura reaction. The number of white blood cell ranged from $5.49–68.93 \times 10^9$/L with mean $16.95 \pm 9.34 \times 10^9$/L; 37 of 126 (29.4%) had leucocytosis ($\geq 20 \times 10^9$/L) [14]. The number of lymphocytes ranged from $2.12–58.2 \times 10^9$/L with mean $11.54 \pm 7.09 \times 10^9$/L; 69 of 126 (54.8%) had lymphocytosis ($\geq 10 \times 10^9$/L) [14]. Suspected bacterial co-pathogens were isolated from 13 (10.3%) of the patients, including: *Haemophilus influenzae* in seven, *Moraxella catarrhalis* in four, *Streptococcus pneumonia* in one, and *Klebsiella pneumonia* in one.

3.2. Drug susceptibility testing

Of all 126 *B. pertussis* strains, 95 (75.4%) were highly resistant (MIC >256 mg/L) against erythromycin, azithromycin and clindamycin. Macrolide-resistant *B. pertussis* isolates were identified in four patients who were not prescribed with any previous antibiotics. The MICs of piperacillin against all of the *B. pertussis* isolates were <0.016 mg/L. The MICs of 11 antibiotics against all strains are summarised in Table 1.

3.3. Treatment and outcome

Forty-nine patients were prescribed a single antibiotic, while 77 received two or more antibiotics (one followed by another or two in combination). Coughing was significantly improved in 114 (90.5%) patients, and 98 of 114 (86.0%) guardians reported significantly improved symptoms within 7 days of initiation of treatment. However, 28 of 114 (24.6%) patients experienced a clinical relapse within 10 days of being discharged. Thirty of 32 (93.8%) patients saw improvement in their symptoms, and 24 of 25 (96%) had negative culture results after 2 weeks of therapy with single cefoperazone-sulbactam or piperacillin-tazobactam. Treatment outcomes of the patients given single macrolides, cefoperazone-sulbactam or piperacillin-tazobactam are summarised in Table 2. More patients treated with macrolides experienced a relapse of symptoms ($P = 0.009$) and had positive culture results (on day 14, $P = 0.003$) than those treated with cefoperazone-sulbactam or piperacillin-tazobactam. Information of the patients with macrolide-resistant or macrolide-sensitive *B. pertussis* is shown in Table 3.

4. Discussion

Pertussis is a notifiable disease, and it is usually clinically diagnosed in China because laboratory methods – such as culture, PCA or ELISA – are not routinely used. The current institute is one of the few hospitals where culture for *B. pertussis* is available. The pertussis patients were culture confirmed in the present study, and most of the patients were aged <6 months and had not been vaccinated with diphtheria-pertussis-tetanus vaccine; thus, they were at risk of pertussis [6,15,16]. Parents or older siblings in most families experienced coughing prior to the initial onset in patients and were found to carry *B. pertussis*, suggesting that family members carrying the bacteria are the main source of infection [17]. Therefore, when considering prevention strategies for pertussis, it is critical to include approaches that prevent transmission among family members [18]. Maternal immunisation during pregnancy is the primary strategy for newborn pertussis protection [18]. If maternal immunisation is not possible, it is recommended that all individuals in close contact with infants aged <6 months be immunised in line with local health authority guidelines [18]. Significantly lower rates of *B. pertussis* in fathers may be associated with previous antibiotics. Breast-feeding

Table 1
MICs of 11 antimicrobial agents against 126 strains of *Bordetella pertussis* in 2016.

Antimicrobial agent	MIC range (mg/L)	MIC_{50}[a] (mg/L)	MIC_{90}[b] (mg/L)
Erythromycin	<0.016 to >256	>256	>256
Azithromycin	<0.016 to >256	>256	>256
Clindamycin	0.064 to >256	>256	>256
SXT	0.023–32	0.19	0.38
Ampicillin	0.023–0.5	0.125	0.25
Ampicillin-sulbactam	0.023–0.25	0.094	0.19
Piperacillin	<0.016	<0.016	<0.016
Cefuroxime	2–16	6	12
Ceftriaxone	0.016–0.75	0.094	0.19
Cefoperazone-sulbactam	<0.016–0.094	0.023	0.047
Meropenem	0.016–0.094	0.032	0.064

SXT, Trimethoprim-sulfamethoxazole.
[a] MIC_{50}, MIC at which 50% of the isolates tested are inhibited.
[b] MIC_{90}, MIC at which 90% of the isolates tested are inhibited.

Table 2
Outcome of the 47 culture-confirmed pertussis patients treated with single macrolides, cefoperazone-sulbactam or piperacillin-tazobactam.

Antibiotics prescribed	Cases (n)	Symptoms improved % (n)	Clinically relapsed % (n)	Positive result of post-treatment culture % (n)		
				Day 7	Day 14	Day 21
Macrolides[a]	15	86.7% (13)	6	60.0% (6/10)	50.0% (4/8)	40.0% (2/5)
Cefoperazone-sulbactam	21	90.5% (19)	3	36.8% (7/19)	7.1% (1/14)	7.1% (1/14)
Piperacillin-tazobactam	11	100.0% (11)	0	27.3% (3/11)	0.0% (0/11)	0.0% (0/11)
Total	47	91.5% (43)	9	40.0% (16/40)	15.2% (5/33)	10.0% (3/30)

Note: Fewer patients treated with cefoperazone-sulbactam or piperacillin-tazobactam experienced a relapse of symptoms (Fisher's exact test, $P = 0.009$) and positive culture results (on day 14, Fisher's exact test, $P = 0.003$) than those treated with macrolides. There were four patients whose symptoms did not improve, although their post-treatment culture results switched to negative on day 7. Two of the three patients with positive post-treatment culture results on day 21 clinically relapsed.

[a] Nine with erythromycin and six with azithromycin; five of the 15 patients were infected with macrolide-sensitive *B. pertussis*; five of the six patients whose symptoms clinically relapsed were infected with macrolide-resistant *B. pertussis*. Thirty-three patients whose symptoms did not improve after being treated with macrolides for >7 days, switched to β-lactams or prescribed with β-lactams in combination were not included in the macrolide group.

Table 3
Comparison of information of patients infected with macrolide-resistant *Bordetella pertussis* and macrolide-sensitive strains.

	Macrolide-sensitive (31)	Macrolide-resistant (95)	χ^2, t or Z value	P-value
Male (n)	15	53	2.48	0.115
Age (months, range, median)	1–41, 2.9	0.8–109, 4.1	$Z = 2.44$	0.015
History of cough (d)	12.8 ± 7.6	17.5 ± 12.0	$t = 2.06$	0.012
Previous antibiotics (d, mean)	5.5 ± 4.7	8.2 ± 5.7	$t = 2.38$	0.018
Previous macrolides (d, range, median)	0–6, 1	0–14, 4	$Z = 4.71$	0.000

mothers who contract infections often decide not to take antibiotics, while fathers will take antibiotics.

The seasonal pattern of pertussis may be different all over the world [5]. The highest incidence occurred from April to August in Zhejiang Province, China. *Bordetella pertussis* strains could be isolated from patients with a long history of coughing (as long as 2 months), even in those with antibiotic treatment, so culture should be routinely used for diagnosis of pertussis. *B. pertussis* is an upper respiratory tract pathogen. However, pneumonia was observed in 47 of 126 (37.3%) patients (85.1% of the 47 were infants), indicating that pertussis in infants is serious and may have co-infection with other pathogens [1,2,6]. About half or more of pertussis patients in this study did not have leucocytosis or lymphocytosis, which may be associated with previous treatment with antibiotics.

Antibiotics are administered to pertussis patients not only to prevent further transmission [19] but also to reduce the severity of the pertussis symptoms [1,19,20]. Macrolide resistance may be overlooked because culture and antimicrobial susceptibility testing is not routinely performed for *B. pertussis* in many countries. Until now, there has been no standardised screening method or interpretive standards for susceptibility testing of *B. pertussis*. Fortunately, MICs of the quality control strains were within the acceptable ranges recommended by the CLSI, indicating that the results of the Etest were acceptable in the study. Although no MIC interpretive standards for *B. pertussis* could be referred to, there is no doubt that MICs >256 mg/L should be interpreted as high resistance, while those with very low MICs are associated with high sensitivity in vitro. In the present study, 75.4% of all isolates had MICs ≥256 μg/mL and were resistant to erythromycin and azithromycin. This high level of resistance was similar to that observed in other Chinese studies [11,12], which collectively indicate the immense selective pressure for macrolide resistance in *B. pertussis* when it colonises the nasopharynx [10]. Resistant phenotypes have been associated with macrolide treatment failure. Most of the patients in the study were prescribed with macrolides before culturing was performed, indicating that macrolides were inactive in inhibiting *B. pertussis* in vivo as well as in vitro, and macrolide-resistant isolates may be selected for a short duration of colonisation [8]. The results showed that symptoms in some patients infected with macrolide-resistant *B. pertussis* improved when they were treated with macrolides. Further study is needed to determine if macrolides inhibit the production of toxins in the bacteria.

Macrolide-resistant *B. pertussis* carries an A-to-G transition at nucleotide position 2047 of the *23S rRNA* gene [9,12], in a region critical for macrolide binding, which may also affect clindamycin binding. The high rate of resistance of clindamycin was also identified in the present study. Four patients who had never received macrolide treatment were infected with macrolide-resistant strains in this study, which may have been due to a heterogeneous phenotype [10] or the horizontal transmission of resistant colonies.

β-lactams are the most commonly prescribed antibiotics for bacterial infections in children. No treatment of pertussis with β-lactams, such as piperacillin or cefoperazone-sulbactam, has yet been documented. *Bordetella pertussis* is resistant to first-generation cephalosporins, so cephalexin was selected as the supplement in the preparation of *Bordetella*-selective charcoal agar medium. The MIC of cefuroxime in this study showed that the isolates were also resistant to second-generation cephalosporins. The inhibitory activity of ceftriaxone in vitro was much better than that of cefuroxime. Cefoperazone-sulbactam had very low MIC_{90} (0.047 mg/L); it also effectively eliminated *B. pertussis* in the nasopharynx and improved symptoms in most patients treated with it. The most sensitive antibiotic was piperacillin: it had potent activity against *B. pertussis* in vitro and inhibited all isolates at concentrations of <0.016 mg/L. Accordingly, piperacillin-tazobactam significantly improved symptoms and eliminated *B. pertussis* strains in the nasopharynx in all (11/11) treated patients. Fewer patients treated with piperacillin-tazobactam or cefoperazone-sulbactam experienced a relapse of symptoms than the macrolide-treated patients. These results indicate that the two antibiotics may be suitable alternative antibiotics for patients infected with macrolide-resistant isolates and with treatment failure.

The main limitation of this study was that, as a single-centre study, the number of patients treated with single cefoperazone-sulbactam or piperacillin-tazobactam in the course of the disease was small, so the therapeutic effects of these antibiotics remain to be determined via a multicentre study covering more cases. Nevertheless, to identify alternative antimicrobial therapies, antimicrobial

susceptibility testing and post-treatment cultures are still necessary for all patients. No significant difference in MIC was found between ampicillin and ampicillin-sulbactam, which indicates that *B. pertussis* may not produce β-lactamase. Meropenem is a broad-spectrum antibiotic and is not recommended for the treatment of pertussis, although most isolates appeared to have low MICs. Therapy with sensitive antibiotics for a sufficient period is necessary for the successful treatment of pertussis; 14 days of erythromycin is usually recommended. Halperin et al. reported that 7 days of erythromycin is as effective as 14 days for the treatment of *B. pertussis* infections [21]. In the present study, post-treatment culture showed that 7 days of macrolides, piperacillin-tazobactam or cefoperazone-sulbactam was usually not enough to eliminate the strains in the nasopharynx, and a 2-week course of treatment is advised. Piperacillin had potent activity against *B. pertussis* in vitro; however, it was not available for therapy in the study and piperacillin-tazobactam was prescribed instead; this was another weakness of the study. A controlled clinical trial including more patients treated with single piperacillin, cefoperazone, or other antibiotics is scheduled to be launched in the next few years.

In conclusion, macrolide resistance in the *B. pertussis* population is a serious problem in Zhejiang Province, China, and it would be of great concern if these strains spread globally. Piperacillin and cefoperazone-sulbactam had potent activity against *B. pertussis* in vitro, and may be suitable alternative antibiotics for patients infected with macrolide-resistant isolates. A controlled clinical trial is recommended to evaluate the effect of these two antibiotics both clinically and bacteriologically in the treatment of *B. pertussis* infection.

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Competing interests

The authors have no conflicts of interest to declare.

Ethical approval

This study was approved by the Ethics Committee and the Institutional Board of Privacy and Security at the hospital (2016-IRB-014), and was performed under the institution's opt-out passive consent policy.

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