High-resolution melting analysis for the detection of two erythromycin-resistant *Bordetella pertussis* strains carried by healthy schoolchildren in China

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Abstract

Two erythromycin-resistant strains of *Bordetella pertussis* were isolated from nasopharyngeal specimens of two asymptomatic schoolchildren in China. High-resolution melting and sequencing analyses confirmed the homogeneous A2047G mutation in 23S rRNA genes of the two isolates. High-resolution melting (HRM) analysis is a useful assay for the rapid detection of erythromycin-resistant *B. pertussis*. The appearance of erythromycin-resistant *B. pertussis* strains in China is alarming.

Keywords: *Bordetella pertussis*, China, erythromycin-resistant, high-resolution melting analysis

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Introduction

Pertussis is an acute respiratory tract infection, and is mainly caused by *Bordetella pertussis*. The disease primarily affects neonates and infants. An increased incidence of pertussis has been found in adults and adolescents in regions where the coverage of vaccination against pertussis in young children is high. The infected adults and adolescents usually constitute an unrecognized but significant source of infection for neonates and infants. Erythromycin has traditionally been the drug of choice for the treatment and prophylaxis of pertussis. Since 1994, erythromycin-resistant isolates have been occasionally reported [1–3], and they have been isolated from infants with severe symptoms. In China, the primary choice of drug for the treatment and prophylaxis of pertussis is erythromycin. In this article, we describe, for the first time, two *B. pertussis* strains isolated from asymptomatic schoolchildren and shown to be resistant to erythromycin.

We conducted a cross-sectional study on pertussis seroprevalence and *B. pertussis* carriage status among healthy adolescents in Shandong province, China, during the period July–September 2011. In this province, DTaP or DTwP vaccine is administered in the third, fourth and fifth months of life, and a booster dose of DTaP vaccine is given at 18–24 months. Booster vaccinations in older children and adolescents are not given in China. During the study, a total of 150 nasopharyngeal swabs were taken from identical subjects for *B. pertussis* culture and PCR. Of thse, two were culture-positive and seven were PCR-positive. Two erythromycin-resistant *B. pertussis* strains were isolated from healthy adolescents without coughing for at least 3 months before blood sampling. They were an 11-year-old boy and a 9-year-old boy, both without cough, sore throat, headaches, or fever symptoms, and they did not develop any respiratory symptoms during a 3-month follow-up. They had completed injection of DTwP vaccine in childhood, according to the national immunization programme, and lived in the same county. None of their household contacts had a history of cough in the 3 months before the study started. Moreover, the two children and their households stated that they had not taken any antibiotics before entering the study. Nasopharyngeal swabs were obtained from the two children, but not from their household members. Two nasopharyngeal swabs that were culture-positive for *B. pertussis* had their positivity confirmed by PCR. The culture plates contained charcoal agar supplemented with 10% defibrinated sheep blood. The identification of two isolates (*Bp*h201101 and *Bp*h201102) was based on biochemical reactions and slide agglutination with specific *B. pertussisis* antisera. Susceptibility testing was performed for erythromycin with Etest gradient strips and a 15-μg erythromycin disk on charcoal agar. The MIC of erythromycin was >256 mg/L for both. Two isolates showed growth confluent to the disk.

The two isolates harboured an allele combination of *ptxA1*/*ptxP1*/*tcfA2*. However, the *prn* allele of the two isolates could not be identified by PCR with the primers and protocol recommended for genotyping [4]. The two isolates had the pulsed-field gel electrophoresis profiles BpFINR9 and BpSR23;

they belonged to the same cluster (cluster III), and resembled those strains circulating in Europe before the 1990s [5]. *Bp*h201101 and *Bp*h201102 were serotype Fim2 and Fim2,3, respectively. PCR-based sequencing was used to determine whether the resistance to erythromycin was caused by a mutation of the 23S rRNA gene of *B. pertussis*, as described by Bartkus *et al.* [1]. The sequences of an approximately 521-bp PCR fragment were determined for *Bp*h201101 and *Bp*h201102, together with two erythromycin-susceptible control strains of *B. pertussis* ATCC 9797 (18323) and the Chinese vaccine strain 58003. An A→G transition homozygous mutation was found at position 2047 of the 23S rRNA gene of the two clinical isolates (Fig.1b).

High-resolution melting (HRM) analysis is a powerful technique for the detection of mutations in double-stranded DNA samples. As compared with sequencing, HRM is rapid, and the cost of reagents is low when a number of samples are analysed. HRM employs saturating double-stranded DNA-binding dyes during the PCR amplification, and denaturation of the PCR amplicons is monitored in real time. Sequence variants are detected from differences in the melting profiles between test and reference DNAs [6]. Therefore, we developed a PCR-based HRM method for specific identification of the A2047G mutation of the 23S rRNA gene of *B. pertussis*. Primers were designed to amplify fragment of nucleotides 1930–2098 flanking the mutation (Table 1). DNAs extracted from the clinical isolates *Bp*h201101 and *Bp*h201102, the *B. pertussis* reference strain ATCC 9797 and the Chinese vaccine strain 58003 were used. In each PCR reaction (20 μL), 5–30 ng of genomic DNA (5 μL) were included. The melting curve was generated by heating in increments of 2.2°C/s, with the temperature range shown in Table 1. To enhance differentiation of the distinct homozygotes (wild type and A2047G transition), the tested DNA samples were spiked with one-fifth of wild-type ATCC 9797 DNA standard. A clear difference in melting curve shape was observed between DNA samples from homozygous mutation and wild-type strains (Fig. 1a).

In conclusion, *B. pertussis* is a strict human pathogen, with no known animal or environmental reservoir. Asymptomatic *B. pertussis* infection has been reported during outbreaks [7,8]. In China, the use of antibiotics is common for the treatment and prophylaxis of all kinds of suspicious infectious diseases. However, whether the use of erythromycin is higher in the county where the study was performed was not known.

Owing to the passive surveillance of pertussis in China, we do not know the prevalence of *B. pertussis* in schools in this country. To our knowledge, this present study is the first to investigate the prevalence of *B. pertussis* in schools. On the basis of the results of this study, the prevalence of *B. pertussis* at the studied school was 4.7% (7/150) by PCR. However, further research with a large number of samples is needed.

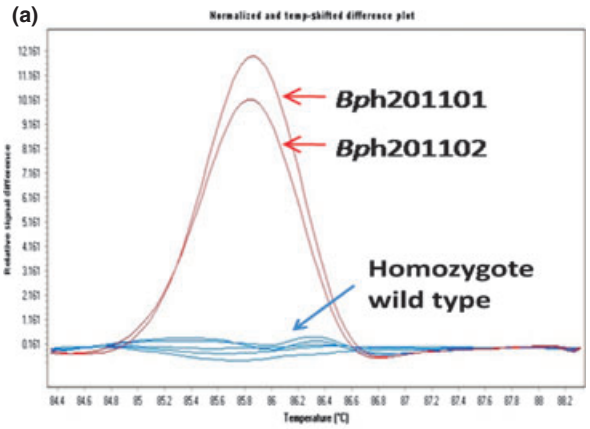

FIG. 1. (a) High-resolution melting analysis for detection of the A2047G mutation of 23S rRNA of the two *Bordetella pertussis* clinical isolates. To enhance differentiation of the distinct homozygotes, the tested DNA samples from the clinical isolates *Bp*h201101 and *Bp*h201102 and the Chinese vaccine strain 58003 were spiked with one-fifth wild-type ATCC 9797 DNA standard. The two red melting curves indicate homozygous mutation in DNA samples of *Bp*h201101 and *Bp*h201102 with spiked ATCC 9797 DNA standard. The blue melting curves indicate homozygous wild-type DNA samples from ATCC 9797 DNA standard, the Chinese vaccine strain 58003 with spiked ATCC 9797 DNA standard, and *Bp*h201101 and *Bp*h201102 clinical isolates without spiked ATCC 9797 DNA standard. The T_m of the homozygous mutations was approximately 85.8°C, and the estimated change in T_m between the homozygous wild type and A2047G transition is approximately 0.24°C [9]. The experiments were repeated four times for both reference strains and clinical isolates. (b) Nucleotide sequences of the 23S rRNA genes of the two erythromycin-resistant *B. pertussis* isolates *Bp*h201101 and *Bp*h201102 and the two erythromycin-sensitive reference strains ATCC 9797 and Chinese vaccine strain 58003. The A→G transition mutation at position 2047 is indicated as bold type.

Primary culture of *B. pertussis* from clinical samples is not sensitive, and takes up to 7 days to obtain a clear result, which restricts rapid detection by phenotypic analyses. The real-time PCR and HRM analysis developed in this study has proven to be sensitive and specific for detecting the mutation responsible for erythromycin resistance in *B. pertussis*. Moreover, the method is rapid and suitable for molecular surveillance of drug resistance in *B. pertussis*.

TABLE 1. Primer sequences and PCR conditions used for high-resolution melting (HRM) analysis for detection of erythromycin-resistant *Bordetella pertussis* isolates

Primer name	Primer sequence (5′–3′)	Amplicon size	PCR conditions[a]	HRM conditions[b]
1930F1 2098R1	CCTGCACGAATGGCGTAA CCTCCCACCTATCCTACAC	168 bp	95°C 2 min 95°C 10 s 60°C 15 s 72°C 10 s	95°C 1 min 40°C 1 min 70°C 1 s 95 continuous

[a]A total of 40 cycles were used.
[b]For HRM conditions, the acquisition mode at 95°C was set as continuous.

B. pertussis carries three copies of the 23S rRNA gene. Using a combined PCR–restriction fragment length polymorphism methodology, Bartkus *et al.* [1] were able to differentiate heterozygous erythromycin-resistant strains from those with homozygous mutation. It remains to be shown whether this PCR-based HRM method can also identify heterozygous erythromycin-resistant strains and possible new mutations other than the A2047G transition.

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Transparency Declaration

The authors declare no conflicts of interest.

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