Direct Detection of Erythromycin-Resistant *Bordetella pertussis* in Clinical Specimens by PCR

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Resistance of *Bordetella pertussis* to erythromycin has been increasingly reported. We developed an allele-specific PCR method for rapid detection of erythromycin-resistant *B. pertussis* directly from nasopharyngeal (NP) swab samples submitted for diagnostic PCR. Based on the proven association of erythromycin resistance with the A2047G mutation in the 23S rRNA of *B. pertussis*, four primers, two of which were designed to be specific for either the wild-type or the mutant allele, were used in two different versions of the allele-specific PCR assay. The methods were verified with results obtained by PCR-based sequencing of 16 recent *B. pertussis* isolates and 100 NP swab samples submitted for diagnostic PCR. The detection limits of the two PCR assays ranged from 10 to 100 fg per reaction for both erythromycin-susceptible and -resistant *B. pertussis*. Two amplified fragments of each PCR, of 286 and 112 bp, respectively, were obtained from a mutant allele of the isolates and/or NP swab samples containing *B. pertussis* DNAs. For the wild-type allele, only a 286-bp fragment was visible when the allele-specific PCR assay 1 was performed. No amplification was found when a number of non-*Bordetella* bacterial pathogens and NP swab samples that did not contain the DNAs of *B. pertussis* were examined. This assay can serve as an alternative for PCR-based sequencing, especially for local laboratories in resource-poor countries.

Pertussis has resurged in many countries. Macrolides, especially erythromycin, are considered the first-choice antibiotics for treatment of pertussis and postexposure prophylaxis (1). The first erythromycin-resistant *Bordetella pertussis* was discovered in 1994 (2). Although resistant strains are still rare, they have been reported in several countries, including China (3–7), where macrolide-resistant *B. pertussis* has become prevalent (7).

B. pertussis is a fastidious bacterium. The culture positivity rate is low in immunized populations. The PCR method has been developed and used for diagnosis of pertussis in many laboratories all over the world. However, it is possible that resistant *B. pertussis* strains are missed due to failure of the culture and when culture is not performed (8).

Several studies have proven the association between macrolide resistance and the A2047G mutation in the 23S rRNA of *B. pertussis* (3–7). Based on the molecular mechanism identified, methods have been developed for detection of the point mutation in 23S rRNA of *B. pertussis* in order to study the susceptibility to macrolides when *B. pertussis* isolates are available (3, 5). In a previous study, we developed a PCR-based sequencing method for identification of the A2047G mutation in cultured *B. pertussis* isolates and clinical nasopharyngeal (NP) specimens (7).

Although the sequencing is widely used, it is not available or accessible everywhere because it takes longer to obtain the results in many of the local laboratories, especially in resource-poor countries. In this study, we aimed to develop a simple allele-specific PCR method for direct detection of erythromycin-resistant *B. pertussis* from clinical specimens submitted for diagnostic PCR.

MATERIALS AND METHODS

Bacteria strains and clinical samples. Fourteen erythromycin-resistant *B. pertussis* strains with the confirmed mutation A2047G and 2 susceptible strains without the mutation were used for this study (9). The 16 clinical strains were isolated between 2012 and 2013 during a prospective study conducted in Xi'an, China (7, 9). They were isolated throughout the study period and not from local small outbreaks. Erythromycin MICs ranged

from 0.023 to >256 μg/ml, and all 14 erythromycin-resistant strains had MICs of >256 μg/ml (9). During the prospective study, NP swab samples were taken from 313 patients with suspected pertussis infection and tested for culture and PCRs (targeting IS*481* and *ptx*-Pr). The age of these patients was 6 days to 11 years (median, 3 months). Of the 313 NP samples, 16 (5.1%) and 168 (53.7%) were positive for culture and IS*481* PCR, respectively. Of the 168 samples positive for IS*481* PCR, 122 and 100 were positive for *ptx*-Pr and 23S rRNA PCRs, respectively. All 100 samples positive for 23S rRNA PCR were also positive for *ptx*-Pr PCR. The primers designed for the 23S rRNA PCR were based on the sequence of domain V of the 23S rRNA gene of the Chinese *B. pertussis* vaccine strain CS (GenBank accession number CP002695.1). The sequences of the primers were previously reported (7), and the sequencing was performed for the PCR-positive products using the forward primer with the BigDye Terminator v31 cycle sequencing kit on ABI3730xl (Applied Biosystems, Carlsbad, CA) from Life Technology Corporation (Shanghai, China). Of the 100 NP samples tested by sequencing, 15 had the wild-type allele and 85 had the mutant allele A2047G. In addition, 100 NP swab samples testing negative for culture, IS*481* PCR, and *ptx*-Pr PCR were selected and used to evaluate the performance of the 2 allele-specific PCR methods. All DNA samples were stored at −20°C.

The ATCC reference strains and clinical isolates *B. pertussis* ATCC 9797, *Bordetella parapertussis* ATCC 15311, *Bordetella bronchiseptica* ATCC 4613, *Klebsiella pneumonia* ATCC 70060, *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 29213, the erythromycin-resistant *B.*

TABLE 1 Primers used in this study

Primer	Sequence (5′ to 3′)	Source or reference
FP	GTGATGGGGTGCAAGCTCTT	This study
RP	TCTGGCGACTCGAGTTCTGC	7
MP[a]	ATCTACCCGCGGCTAGACAG**G**	This study
WP[a]	ATCTACCCGCGGCTAGAC<u>A</u>G**A**	This study

[a] The primers MP and WP contained a specific mismatch A (underlined) at the 3′ end that did not complement the published sequence of 23S rRNA of *B. pertussis* (see Fig. 1). The G (boldfaced) at the 3′ end of the MP primer was complementary to 2047G of erythromycin-resistant *B. pertussis*, whereas the A (boldfaced) at the 3′ end of the WP primer was complementary to A2047 of erythromycin-sensitive *B. pertussis*.

pertussis strain bp12152, *Neisseria meningitidis*, and *Haemophilus influenzae* were used to determine the specificity and sensitivity of the PCR assays.

Allele-specific PCR assays. The sequences of the four primers used for the two allele-specific PCR assays are shown in Table 1 and Fig. 1. Primers MP and WP contained a specific mismatch A at the 3′ end that did not complement the published sequence of 23S rRNA of *B. pertussis* (Fig. 1). The G at the 3′ end of the MP primer was complementary to 2047G of erythromycin-resistant *B. pertussis*, whereas the A at the 3′ end of the WP primer was complementary to A2047 of erythromycin-sensitive *B. pertussis*. In each of the two PCRs, three primers were used. For assay 1, primers FP, MP, and RP were included; for assay 2, primers FP, WP, and RP were included. The length of the PCR products based on the forward primer FP and reverse primer RP was 286 bp, whereas the length of the PCR products based on the forward primer MP or WP and reverse primer RP was 121 bp. The working principle of the two PCR assays is described below. In assay 1, the two mismatches at the 3′ end of primer MP would guarantee the absence of PCR amplification if the sequence of erythromycin-sensi-

tive *B. pertussis* DNA is used as the target. Therefore, there would be only one PCR product of 286 bp. However, if the sequence of erythromycin-resistant *B. pertussis* DNA is used as the target, PCR amplification of 121 bp would also be present, since there was only one mismatch at the 3′ end of primer MP. In assay 2, the two mismatches at the 3′ end of primer WP would guarantee the absence of PCR amplification if the sequence of erythromycin-resistant *B. pertussis* DNA is used as the target. Therefore, there would only be one PCR product of 286 bp. However, if the sequence of erythromycin-sensitive *B. pertussis* DNA is used as the target, PCR amplification of 121 bp would also be present, since there was only one mismatch at the 3′ end of the primer WP. DNA extraction of the reference strains was performed by using the DNA minikit (Qiagen). For allele-specific PCR assay 1, the 20 μl total volume of PCR mixture contained 10 μl of HotStar *Taq* master mix; 0.5 μM each of primers FP, MP, and RP; and 2 μl of DNA extracts. Amplification was performed in PTC200 thermal cycler (Bio-Rad, CA) with the following conditions: 95°C for 15 min, 35 cycles of 94°C for 1 min, 60°C for 30 s, 72°C for 30 s, and the final extension with 72°C for 10 min. PCR products were resolved on 2% agarose gel, stained with GelRed, and photographed with GelDox XR (Bio-Rad).

For allele-specific PCR assay 2, the total volume, reaction mixture, and conditions were the same as those of PCR assay 1, except for primer MP, which was replaced by primer WP.

The work flow for determining the A to G mutation of the 2047 nucleotide of 23S rRNA from the clinical NP samples submitted for diagnostic PCR is illustrated in Fig. 2. It is known that *B. pertussis* carries three copies of the 23S rRNA gene. Heterozygous erythromycin-resistant strains have been reported (3). If the information on heterogeneous susceptibility to erythromycin or coinfection with erythromycin-susceptible *B. pertussis* is needed, the allele-specific PCR assay 2 should be performed.
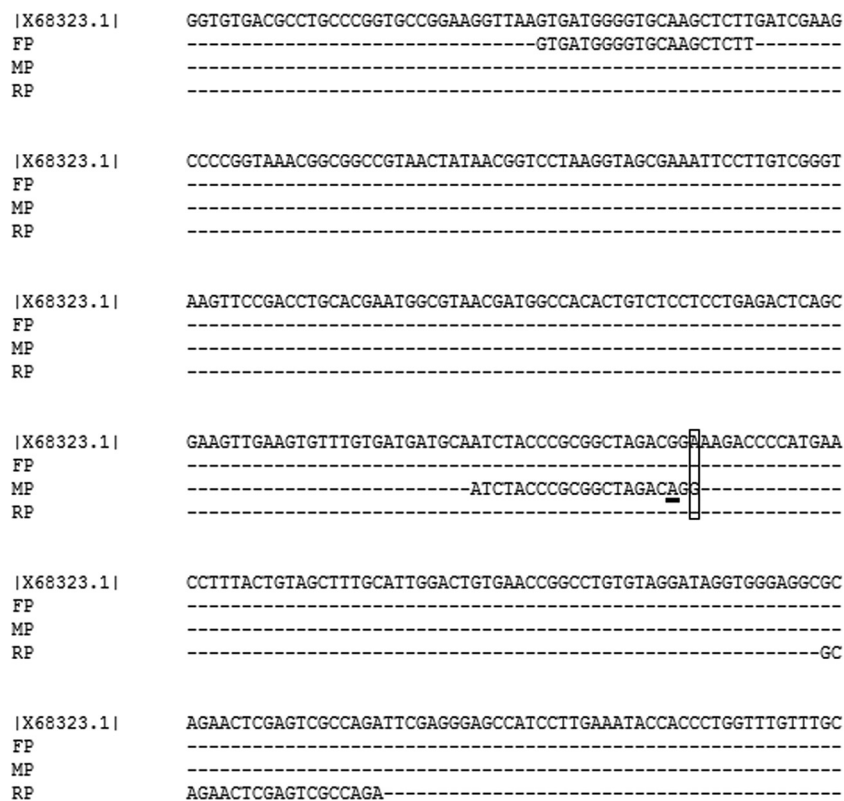
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|X68323.1|    GGTGTGACGCCTGCCCGGTGCCGGAAGGTTAAGTGATGGGGTGCAAGCTCTTGATCGAAG
FP            --------------------------------GTGATGGGGTGCAAGCTCTT--------
MP            ------------------------------------------------------------
RP            ------------------------------------------------------------


|X68323.1|    CCCCGGTAAACGGCGGCCGTAACTATAACGGTCCTAAGGTAGCGAAATTCCTTGTCGGGT
FP            ------------------------------------------------------------
MP            ------------------------------------------------------------
RP            ------------------------------------------------------------


|X68323.1|    AAGTTCCGACCTGCACGAATGGCGTAACGATGGCCACACTGTCTCCTCCTGAGACTCAGC
FP            ------------------------------------------------------------
MP            ------------------------------------------------------------
RP            ------------------------------------------------------------


|X68323.1|    GAAGTTGAAGTGTTTGTGATGATGCAATCTACCCGCGGCTAGACGG[A]AGACCCCATGAA
FP            ------------------------------------------------------------
MP            --------------------------ATCTACCCGCGGCTAGACAG‾G-------------
RP            ------------------------------------------------------------


|X68323.1|    CCTTTACTGTAGCTTTGCATTGGACTGTGAACCGGCCTGTGTAGGATAGGTGGGAGGCGC
FP            ------------------------------------------------------------
MP            ------------------------------------------------------------
RP            ----------------------------------------------------------GC


|X68323.1|    AGAACTCGAGTCGCCAGATTCGAGGGAGCCATCCTTGAAATACCACCCTGGTTTGTTTGC
FP            ------------------------------------------------------------
MP            ------------------------------------------------------------
RP            AGAACTCGAGTCGCCAGA------------------------------------------
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FIG 1 Partial sequence of the 23S rRNA gene of *Bordetella pertussis*, showing the positions of primers FP, MP, and RP used in the allele-specific PCR. Consensus bases are shown with dashes, and the mismatched bases in the primer are indicated by box and the underline. The position of primer WP is the same as that of primer MP, and the sequence of primer WP is shown in Table 1.
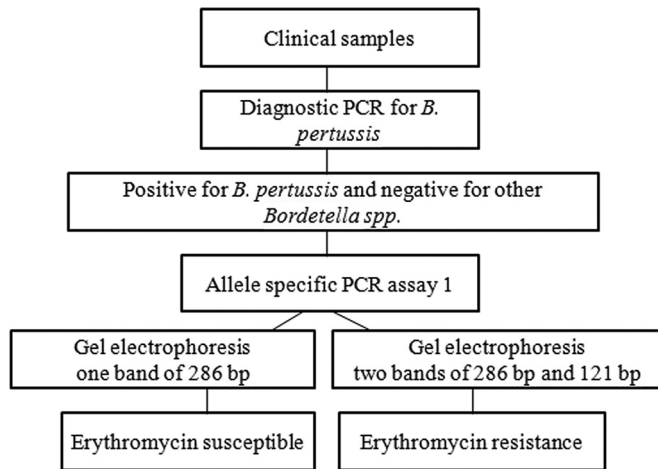
FIG 2 Work flow for determining the A to G mutation of the 2047 nucleotide of 23S rRNA from clinical samples submitted for diagnostic PCR used.

Allele specfic PCR assay 1   **Allele specfic PCR assay 2**

FIG 3 Gel electrophoresis of the amplified products by allele-specific PCR assays 1 and 2. Lanes: M, 100-bp ladder; WT, ATCC 9797 *B. pertussis* reference strain (erythromycin sensitive); MT, clinical *B. pertussis* isolate (erythromycin resistant); S, clinical NP swab; N, negative control of PCR.

Detection limits and specificity assay. The detection limits of the two allele-specific PCR assays were measured using serially diluted DNA solutions of an erythromycin-susceptible strain of *B. pertussis* ATCC 9797 and an erythromycin-resistant strain of bp12152. The concentrations tested ranged from 1 fg/μl to 1 ng/μl.

The specificity of the two PCR assays was determined by using DNA extracts from other *Bordetella* spp., including *B. parapertussis* ATCC 15311, *B. bronchiseptica* ATCC 4613, *B. holmesii* FR4020, *B. petrii* FR3497, *B. avium* FR3815, and *B. hinzii* FR3756 and from other bacterial species mentioned above. The concentrations used were 1 ng/μl.

RESULTS

Allele-specific PCR assays. Allele-specific PCR assay 1. As designed, a single band of a 286-bp fragment was visible when the PCR was tested with the DNA extracts from erythromycin-susceptible *B. pertussis* (Fig. 3). In contrast, two bands of 286 and 121 bp were obtained with the DNA extracts from erythromycin-resistant *B. pertussis*.

Allele-specific PCR assay 2. For erythromycin-susceptible *B. pertussis*, two bands of 286 and 121 bp were obtained. Only one band of 286 bp was obtained when erythromycin-resistant *B. pertussis* was tested (Fig. 3).

Detection limits and specificity. The detection limits of the two PCR assays ranged from 10 to 100 fg per reaction for erythromycin-susceptible and -resistant *B. pertussis*. No amplification was detected when DNAs isolated from *K. pneumoniae*, *E. coli*, *S. aureus*, *N. meningitidis*, and *H. influenzae* were tested. However, except for *B. holmesii*, all *Bordetella* spp. tested produced the 286-bp fragment.

Evaluation of the allele-specific PCR assays by clinical samples submitted for diagnostic PCR. Of the 100 NP samples tested by PCR-based sequencing, 15 were found to have a wild-type allele, and 85 were found to have the mutant allele A2047G (Table 2). Ninety-five of these samples were positive according to allele-specific PCR assays, and they contained 16 culture-positive samples. The reasons the 5 remaining samples were negative by the allele-specific PCR assays are unknown. One explanation may be that these DNA samples contained a limited amount of *B. pertussis* DNA. Another explanation may be that the samples were already used several times for different diagnostic PCR tests and PCR-based sequencing. In addition, we cannot exclude the possibility
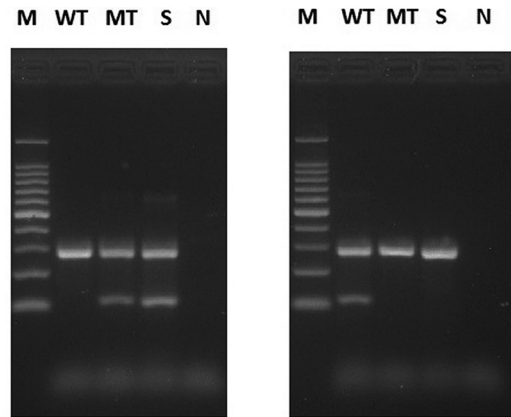
that there might be competitive binding of the three primers used in one PCR, especially when a limited amount of the target DNAs were included.

Moreover, when the other 100 NP samples that were negative for the diagnostic (*ptx*-Pr) PCR were tested with the allele-specific PCR assays, they all tested negative.

DISCUSSION

Macrolides, especially erythromycin, are used as the first-choice antibiotics for treatment and prevention of pertussis transmission. However, the susceptibility test cannot be done without bacterial isolates. In this study, we developed a simple PCR method for detection of erythromycin-resistant *B. pertussis* directly from clinical NP samples. This method is specific, rapid, and easily undertaken compared to currently available methods. Moreover, it is suitable for certain local laboratories, especially those in resource-poor countries where the sequencing technology is not available or where it takes a longer time to obtain the sequencing results. It is well known that the correct and early use of antibiotics is im-

TABLE 2 Comparison of the results from PCR-based sequencing and the allele-specific PCR assay in determination of the A2047G mutation of 23S rRNA in *B. pertussis* from 200 nasopharyngeal swab samples submitted for diagnostic PCR[a]

Allele-specific PCR assay	PCR sequencing assay			
	Wild type	Mutation type	Negative	Total
Wild type	14[b]	0	0	14
Mutation type	0	81[c]	0	81
Negative	1	4	100	105
Total	15	85	100	200

[a] The 200 nasopharyngeal (NP) swab samples included 100 positive and 100 negative samples tested by diagnostic *ptx*-Pr PCR (7). Of the 100 *ptx*-Pr PCR positive samples, 16 were also culture positive. Of the 16 *B. pertussis* strains, 14 were erythromycin resistant and 2 were erythromycin sensitive.
[b] Containing the 2 NP samples from which *B. pertussis* was isolated and proven to be erythromycin sensitive.
[c] Containing the 14 NP samples from which *B. pertussis* was isolated and proven to be erythromycin resistant.

portant for treatment and prevention, in addition to saving resources.

Antibiotic resistance plays a key role in the (re)emergence of infectious diseases (10). After the first erythromycin-resistant *B. pertussis* was reported, researchers paid more attention to the resistant strains, especially when pertussis resurgence occurred. However, as far as we can discern, antibiotic-resistant *B. pertussis* was only found occasionally in the United States and France (4). Studies carried out in Australia, Taiwan, the United Kingdom, Romania, and Canada showed that *B. pertussis* isolated in these countries was sensitive to erythromycin (11–15). According to recent research in Iran, 2 of 11 *B. pertussis* strains isolated between 2009 and 2010 were macrolide resistant (16). However, in China, macrolide-resistant *B. pertussis* emerged and became common when strains in different regions were tested (5, 17).

In the antibiotic era, bacteria face selective pressure from antibiotics and always evolve to adapt. To our knowledge, there is no molecular mechanism other than the A2047G substitution in the 23S rRNA of *B. pertussis* that has been linked to erythromycin resistance. Our previous study based on the sequencing of 23S rRNA of *B. pertussis* also found no other mutations in the gene studied (7). Therefore, we considered that detection of the specific A to G mutation can provide the same information as that obtained from phenotypic analysis of *B. pertussis* isolates.

In the allele-specific PCR assay, when primers designed to be specific for either wild-type or mutant allele are used, results depend on the absence or presence of amplification. In this study, we found that primer MP contains two mismatches at its 3′ end, and this guarantees the absence of PCR amplification when the sequence of erythromycin-sensitive *B. pertussis* DNA is used as the target. In contrast, primer WP was found to contain two mismatches at its 3′ end, and this guarantees the absence of PCR amplification when the sequence of erythromycin-resistant *B. pertussis* DNA is used as the target. The sequences of the two primers are almost identical, except for the last nucleotide at their 3′ ends. Our results confirm that the last nucleotides of the 3′ ends of primers are crucial for primer binding (18).

So far, the molecular mechanism of *B. pertussis* resistance to erythromycin has been associated with the A2047G substitution in domain V of 23S rRNA (3). It remains to be shown if there is an alternative mechanism that can confer erythromycin resistance. Azithromycin is another commonly used macrolide and more active than erythromycin against *B. pertussis*. In our previous study, we analyzed the MICs of azithromycin against 14 erythromycin-resistant and 2 erythromycin-sensitive strains (9). Although the MICs of azithromycin against the 16 tested strains were generally lower than those of erythromycin, all strains except the 2 that were erythromycin sensitive were also resistant to azithromycin, suggesting that the A2047G mutation in domain V of 23S rRNA of *B. pertussis* might be implicated in azithromycin resistance, at least among the clinical isolates analyzed in this study. Further studies are needed to confirm the association.

The study had some limitations. First, the sequences in 23S rRNA are similar among the *Bordetella* species. As shown in the present study, the allele-specific PCRs may also amplify all except *B. holmesii* of the *Bordetella* species. However, this should not be a problem in clinical practice, since this PCR will be performed after the diagnostic PCR specific for *B. pertussis*. Second, it is known that there are 3 copies of 23S rRNA in the genome of *B. pertussis*. The heterozygote and homozygote variants of 23S rRNA in *B. pertussis* found can confer the heterogeneous susceptibility to erythromycin (3). However, we tested the 100 clinical samples using this allele-specific PCR and PCR-based sequencing and did not find any heterozygote variants, suggesting that the heterozygote variants of 23S rRNA in *B. pertussis* are rare in this study area. In order to determine whether the heterozygote variant of 23S rRNA in *B. pertussis* or the coinfection of erythromycin-susceptible *B. pertussis* exists (19), we suggest performing an additional allele-specific PCR assay 2 when allele-specific PCR assay 1 produces two different fragments.

In conclusion, we developed a simple PCR assay for rapid identification of erythromycin-resistant *B. pertussis* directly from clinical specimens submitted for diagnostic PCR for pertussis. The assay serves as an alternative for PCR-based sequencing, especially for local laboratories in resource-poor countries, where sequencing is difficult to access.

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