Identification of a Mutation Associated with Erythromycin Resistance in *Bordetella pertussis*: Implications for Surveillance of Antimicrobial Resistance

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Erythromycin treatment failures and in vitro resistance of *Bordetella pertussis* have been reported on several occasions in the past few years, but the mechanism of resistance has not been described. One potential mechanism, genetic modification of the erythromycin-binding site on the 23S rRNA of the 50S ribosomal subunit, has been observed in other bacteria. To explore this possibility, we amplified the portion of the 23S rRNA gene encoding the central loop of domain V. DNA sequencing and restriction fragment length polymorphism of the PCR products showed that each of the four erythromycin-resistant *B. pertussis* strains tested contained an A-to-G transition mutation at position 2058 (Escherichia coli numbering) of the 23S rRNA gene. The mutation was not found in seven erythromycin-susceptible isolates tested. Two of the resistant isolates were heterozygous, containing at least one mutant copy and one wild-type copy of the 23S rRNA gene. These results indicate that erythromycin resistance in these strains is likely due to a mutation of the erythromycin-binding site in the 23S rRNA gene. Identification of the resistance mechanism will facilitate development of molecular susceptibility testing methods that can be used directly on clinical specimens in the absence of an isolate.

*Bordetella pertussis*, the causative agent of pertussis, continues to be an important cause of morbidity in the United States. Although the incidence of pertussis has declined since the prevaccine era, the number of annual cases has risen since 1980, with 7,867 cases of pertussis reported nationally in 2000 (3). Antibiotic treatment and prophylaxis play an important role in controlling the spread of pertussis in the United States. Erythromycin eliminates the organism from the respiratory tract and is currently the treatment of choice for patients with confirmed pertussis and their close contacts (1, 8–10, 26; J. W. Bass, Letter, *Lancet* ii:147, 1985).

The first case of erythromycin-resistant *B. pertussis* was identified in Yuma, Ariz., in 1994 (2, 15). To-date, four additional cases of erythromycin-resistant *B. pertussis* have been reported in the United States (7). Most recently, screening of 1,030 isolates of *B. pertussis* revealed an additional five strains exhibiting a heterogeneous erythromycin resistance phenotype (37). Elucidation of the mechanism of resistance is important for identifying appropriate antimicrobial therapy as well as approaches for limiting resistance dissemination. Due to the increased exclusive use of molecular methods, such as PCR, for detection of *B. pertussis*, molecular tests to detect resistance, even in the absence of an isolate, need to be developed.

Erythromycin inhibits nascent peptide elongation by binding to the bacterial 23S rRNA component of the 50S ribosomal subunit (19). The critical recognition site for erythromycin binding is a nucleotide sequence having a structural function critical to the peptidyl transferase activity of the ribosome. This region of the 23S rRNA is typically termed domain V, and the individual nucleotides are enumerated in the order of their occurrence based on the equivalent 23S rRNA sequence of *Escherichia coli*. Resistance to erythromycin may be conferred by a number of different mechanisms (36), but alteration of the erythromycin-binding site, active efflux of the antimicrobial agent, and enzymatic inactivation of the antibiotic are the most common. Some gram-positive agricultural and clinical bacterial isolates alter a key nucleotide involved in erythromycin binding to the 23S rRNA by addition of a methyl group (12). This is accomplished posttranscriptionally through the activity of the *erm* gene methylase on nucleotide A2058 of the 23S rRNA (*E. coli* coordinates). Methylation of A2058 can result in resistance to macrolides (14- to 16-membered rings) other than erythromycin and in cross-resistance to lincosamides and type B streptogramin antibiotics that also bind to nucleotide A2058 (36).

Recently, a chromosomal mutation that alters the erythromycin binding site in domain V has been shown to confer erythromycin-resistance in a number of clinical isolates, including *Helicobacter pylori*, *Propionibacterium* sp., *Mycoplasma pneumoniae*, *Mycobacterium avium*, and *Mycobacterium intracellulare* (16, 18, 21, 24, 32). The most common mutation in these isolates occurs at positions cognate with the nucleotide equivalent to A2058 of the rRNA (*rrn* operon encoding the 23S rRNA and typically confers the lincosamide-streptogramin resistance phenotype (36).

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TABLE 1. *Bordetella pertussis* isolates tested for erythromycin susceptibility

<table>
<thead>
<tr>
<th>Strain</th>
<th>Erythromycin phenotype</th>
<th>Erythromycin MIC(s) (μg/ml)</th>
<th>Mutation</th>
<th>Source (reference)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MN2726</td>
<td>Susceptible</td>
<td>0.064</td>
<td>None (wt)</td>
<td>This study</td>
</tr>
<tr>
<td>MN277</td>
<td>Susceptible</td>
<td>0.064</td>
<td>None (wt)</td>
<td>This study</td>
</tr>
<tr>
<td>MN973</td>
<td>Susceptible</td>
<td>0.064</td>
<td>None (wt)</td>
<td>This study</td>
</tr>
<tr>
<td>MN1286</td>
<td>Susceptible</td>
<td>0.032</td>
<td>None (wt)</td>
<td>This study</td>
</tr>
<tr>
<td>MN1699</td>
<td>Susceptible</td>
<td>0.047</td>
<td>None (wt)</td>
<td>This study</td>
</tr>
<tr>
<td>MN1773</td>
<td>Susceptible</td>
<td>0.047</td>
<td>None (wt)</td>
<td>This study</td>
</tr>
<tr>
<td>MN1893</td>
<td>Susceptible</td>
<td>0.047</td>
<td>None (wt)</td>
<td>This study</td>
</tr>
<tr>
<td>MN2531</td>
<td>Resistant</td>
<td>&gt;256</td>
<td>A2047G</td>
<td>Arizona (2, 22)</td>
</tr>
<tr>
<td>A228&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Resistant</td>
<td>&gt;256&lt;sup&gt;b&lt;/sup&gt;</td>
<td>A2047G</td>
<td>California (14)</td>
</tr>
<tr>
<td>C310&lt;sup&gt;c&lt;/sup&gt;</td>
<td>Resistant</td>
<td>&gt;256&lt;sup&gt;b&lt;/sup&gt;</td>
<td>A2047G</td>
<td>Georgia</td>
</tr>
<tr>
<td>C352&lt;sup&gt;a,c&lt;/sup&gt;</td>
<td>Heterogeneous</td>
<td>0.047, &gt;256&lt;sup&gt;b&lt;/sup&gt;</td>
<td>A2047R</td>
<td>Georgia</td>
</tr>
<tr>
<td>C353&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>Resistant</td>
<td>&gt;256&lt;sup&gt;b&lt;/sup&gt;</td>
<td>A2047G</td>
<td>Georgia</td>
</tr>
</tbody>
</table>

<sup>a</sup> Supplied by the CDC.
<sup>b</sup> MIC originally determined by CDC using methods described previously (7) and retested at MDH for purposes of comparison.
<sup>c</sup> Strain C352 exhibits a heterogeneous phenotype (a mix of sensitive and resistant colonies) with respect to erythromycin resistance (37). Sequencing of the 521-bp PCR fragment resulted in an unresolved base (A or G) at position 2047, indicated by an R in the nucleotide sequence.

**MATERIALS AND METHODS**

**Bacterial strains.** The strains examined in this study and their susceptibilities to erythromycin are listed in Table 1. This list includes isolates of *B. pertussis* submitted to the Minnesota Department of Health (MDH) as part of the Enhanced Pertussis Surveillance Program supported by the Centers for Disease Control and Prevention (CDC) as well as previously identified erythromycin-resistant isolates of *B. pertussis* from the CDC strain collection.

**Determination of MICs.** Standardized susceptibility test methods have not yet been established for *B. pertussis*. Susceptibility testing on study isolates was done for erythromycin, azithromycin, and clarithromycin using Etest gradient strips according to the manufacturer’s instructions (AB Biodisk, Solna, Sweden). Isolates were also tested for susceptibility to erythromycin using a modified Kirby-Bauer disk diffusion method. Isolates were grown for 48 h on charcoal agar medium (Unipath, Ltd., Basingstoke, United Kingdom) supplemented with 10% defibrinated sheep blood and without cephalaxin (CA-C). Cells from the plate were suspended in tryptic soy broth to match a 0.5 McFarland standard using the NCCLS direct colony suspension method (22). The suspension was then swabbed evenly onto the surface of a CA-C plate (150 by 100 mm) and allowed to dry before applying a 15-μg erythromycin disk (Remel, Lenexa, Kans.) or one each of the azithromycin, clarithromycin, and erythromycin Etest strips (AB Biodisk). The plates were placed in plastic bags to prevent moisture loss and incubated at 35°C in ambient air. Zone size and MICs were read after 48 to 72 h of incubation. Standardized interpretation criteria currently do not exist for *B. pertussis*; however, disk diffusion zone sizes of at least 24 mm have been associated with susceptibility (7), and the MICs of erythromycin against susceptible strains reportedly range from 0.02 to 0.12 μg/ml (7, 13).

**Template DNA preparation.** DNA was prepared from *B. pertussis* cultures by transferring cells from a CA-C plate into 200 μl of DNAzol (Molecular Research Center, Cincinnati, Ohio) and boiling the suspension for 15 min. Following centrifugation to remove cellular debris, 200 μl of the sample was added directly to a Qiagen column (Qiagen Inc., Valencia, Calif.) spin column, centrifuged, washed, and eluted according to the manufacturer’s instructions.

**PCR.** We used the method of Sutcliffe et al. (26) for detecting erythromycin methylases among *B. pertussis* test strains by PCR and failed to detect any products. To look for mutations in the 23S ribosomal DNA (rDNA) that may be associated with erythromycin resistance in *B. pertussis*, PCR primers were used to amplify a region of the central loop of domain V containing E. coli-equivalent nucleotides A2058 and A2059. These represent the most common sites for mutations conferring erythromycin resistance. Primers 1907U (5’TTCCTGTGTG

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B resistance phenotype (36). In *H. pylori* and propionibacteria, similar mutations at the adjacent nucleotide, equivalent to A2059, resulted in macrolide resistance but only moderate resistance to lincosamides and no resistance to type B streptogramins (24, 35).

The mechanism of *B. pertussis* resistance to erythromycin has not yet been identified, although a previous study suggested that methylation of the 23S rRNA binding site is not involved (2). This result, and the complexity and metabolic cost inherent in adapting an active erythromycin efflux system, prompted us to evaluate the 23S rRNA sequence for mutations that might disrupt erythromycin binding. We report here the identification of the first erythromycin-resistant isolate of *B. pertussis* in Minnesota, identify a chromosomal mutation in the gene encoding 23S rRNA in this strain, and associate this mutation with resistance to erythromycin in this and several other erythromycin-resistant strains.

(This work was presented in part at the 41st Interscience Conference on Antimicrobial Agents and Chemotherapy.)

**CASE REPORT**

A 10-year-old girl presented to urgent care on 23 October 1999 with a 2.5-week history of nonproductive episodic cough, sinus congestion, sore throat, headaches, and no fever. Significant underlying medical conditions included a history of familial asthma and phenylketonuria. She had a history of vaccinations included only diphtheria and tetanus toxoids due to a medical contraindication. Following a presumptive diagnosis of pharyngitis and bronchospasm, treatment with azithromycin was begun on 23 October 1999. Nine days later, due to continued cough and increased sinus congestion, the patient returned to her health care provider and treatment was changed to cephalexin. The patient returned to her health care provider on 5 November 1999 at which time, the health care provider noted the paroxysmal cough with inspiratory whoop, changed to cephalexin. The patient returned to her health care provider and treatment was continued cough and increased sinus congestion, the patient made a presumptive diagnosis of pertussis. Treatment with erythromycin was begun on 23 October 1999. Nine days later, due to continued cough and increased sinus congestion, the patient returned to her health care provider and treatment was changed to cephalexin. The patient returned to her health care provider on 5 November 1999 at which time, the health care provider noted the paroxysmal cough with inspiratory whoop.

No subsequent cases of pertussis were identified among her classmates or household contacts.
RESULTS

Determination of erythromycin MIC. Antimicrobial susceptibility testing was conducted on 573 *B. pertussis* isolates obtained by the MDH from January 1997 through December 1999 as part of the CDC-supported Enhanced Pertussis Surveillance Program. The MIC of erythromycin against 572 *B. pertussis* isolates obtained ranged from 0.016 to 0.094 μg/ml. One isolate, designated MN2531, was found to be resistant to erythromycin; the Etest MIC was >256 μg/ml, and the diameter of the disk diffusion zone was 6 mm. These values are similar to those found in other erythromycin-resistant isolates of *B. pertussis* (Table 1) (7). We determined the MICs for additional macrolides in the erythromycin-resistant isolate from Minnesota and a subset (n = 264) of the 572 erythromycin-susceptible isolates. For the susceptible strains, MICs of azithromycin were 0.016 to 0.125 μg/ml and those of clarithromycin were 0.023 to 1 μg/ml. The Etest MICs of azithromycin and clarithromycin were >256 μg/ml for erythromycin-resistant strain MN2531.

Strain C352 was found by the CDC to have a heterogeneous pattern of resistance to erythromycin in the presence of a 50-μg erythromycin disk, exhibiting both sensitive and resistant populations of cells. A representative erythromycin-resistant isolate, C353, was purified to homogeneity and found to be stably resistant to erythromycin. The sensitive subpopulation of cells was diluted to extinction, and multiple colonies were picked and restested with the erythromycin disk. Following two rounds of purification, however, all isolates still expressed the heterogeneous phenotype, spontaneously giving rise to resistant strains at a high frequency. Retesting of isolates at the MDH with erythromycin disks and Etest strips yielded similar results.

DNA sequencing results. PCR amplification and DNA sequencing were used to determine whether resistance to erythromycin in *B. pertussis* was due to a mutation in the 23S rRNA gene. The sequence of the putative erythromycin-binding site of the *B. pertussis* 23S rRNA gene was determined by sequencing approximately 400 nucleotides of the 521-bp PCR fragment amplified from MN2531 and an erythromycin-susceptible strain of *B. pertussis*, MN2726, having the same chromosomal profile by pulsed-field gel electrophoresis. The DNA sequences from these two isolates were compared to each other and to that of the GenBank (X68323) and Sanger Center sequences of the *B. pertussis* Tohama strain. An A-to-G transition mutation was found in the erythromycin-resistant strain MN2531 at nucleotide position 2027 (GenBank sequence) or 2047 (Sanger sequence) of the 23S rRNA gene. We noted a discrepancy between the Sanger Center and GenBank sequences of the *B. pertussis* Tohama strain 23S gene. Because our sequence data matched that of the Sanger Center, we propose to assign numbering of the A-to-G mutation in *B. pertussis* according to the position of the nucleotide in the Sanger sequence. Position 2047 of the *B. pertussis* 23S sequence is homologous to position A2058 in *E. coli*. Sequencing was similarly performed on previously isolated erythromycin-resistant strains submitted to the CDC. CDC strains A228, C310, and C353 all contained the A2047G transition mutation (Table 1). Sequencing of the heterogeneous strain, C352, yielded a mixed base, A or G, at position 2047. *B. pertussis* has been reported to contain three copies of the *rrn* operon (20). Our sequencing results indicate that C352 is heterozygous, harboring at least one mutant copy of the *rrn* operon and one copy of the wild-type allele. C353, a homogeneously resistant isolate derived from C352, appeared to be homozygous with regard to the A2027G mutation. It appears likely that the heterogeneous phenotype of C352 is due to heterozygosity of the *rrn* operon and that the stably resistant phenotype of C353 is due to incorporation of the A2047G mutation into all copies of the *rrn* operon by homologous recombination.

PCR-RFLP results. The A-to-G transition at position 2047 in erythromycin-resistant strains of *B. pertussis* is predicted to result in the addition of a *BbsI* restriction site. To confirm the presence of the transition mutation (G2047) in erythromycin-resistant strains versus the wild-type sequence (A2047) in susceptible strains, the 521-bp PCR amplicon of 23S rDNA was digested with *BbsI*. Digestion with *BsaI* was also performed to use as a screening method to detect A2048G mutations, the next most common site for mutations conferring erythromycin resistance, as well as to serve as a restriction digestion control. As shown in Fig. 1, each of the erythromycin-resistant strains tested were cleaved by *BbsI*, yielding the expected products of 128 and 393 bp. This is consistent with the DNA sequencing data, indicating an A-to-G transition mutation at nucleotide 2047 in these strains. *BbsI* failed to cleave the 521-bp amplicons from the seven erythromycin-susceptible clinical isolates tested. *BsaI* digestion yielded the expected fragments (197 and 324 bp) from all isolates, indicating the presence of a wild-type base at position A2048. Amplicons from MN2531 and C353 cut to completion with *BbsI*; however, isolates A228 and C352 exhibited residual DNA corresponding to the uncut 521-bp amplicons, indicating the presence of at least one wild-type copy of the *rrn* operon in these isolates. This result was expected in the case of C352, which exhibited a mixed base at position 2047; however, the sequence data for A228 did not
indicate heterozygosity. In addition, the phenotype of A228, which is homogeneously resistant to erythromycin, differs from that of the heterogeneously resistant C352. Repeated efforts to eliminate the undigested band through increased duration of restriction digestion and to exclude the possibility of a hetero-
geneous culture by isolation and analysis of single colonies were unsuccessful, indicating that the presence of the undi-
gested band was likely due to the presence of a wild-type copy of the

\[ rrn \]

operon in these strains. One possible explanation for the different phenotypes of C352 and A228 is that the mutation is present in only one copy of the \( rrn \) operon in C352 and in two copies of the \( rrn \) operon in A228.

**Analysis of heterozygotes.** Because we observed a difference in the relative intensities of the cut and uncut fragments generated from strains A228 and C352, seen in Fig. 1, we performed quantitative densitometric analysis of the 521-bp uncut fragment and the cut fragments of 128 and 393 bp to estimate the amount of wild-type copy of the \( rrn \) operon in these strains. One possible explanation for the different phenotypes of C352 and A228 is that the mutation is present in only one copy of the \( rrn \) operon in C352 and in two copies of the \( rrn \) operon in A228.

**DISCUSSION**

The data presented in this study suggest that erythromycin resistance in the \( B. pertussis \) strains isolated to date is due to an A-to-G transition mutation at position 2047 of the Sanger Center sequence of the \( B. pertussis \) 23S rRNA gene. This position is analogous to position 2058 in \( E. coli \). The mutation was found in each of four erythromycin-resistant \( B. pertussis \)
isolates but was absent in seven susceptible strains tested. Position A2058 of the 23S rRNA is believed to be the key nucleotide involved in macrolide interaction with the ribosome (19). The transition from A to G at the position corresponding to 2058 is the most frequent of the mutations associated with macrolide resistance in clinical isolates, including *H. pylori* (11, 30–32), *Mycobacterium* spp. (17, 18, 21, 34), *M. pneumoniae* (16), and propionibacteria (24). Bias toward the A2058G-equivalent mutation has been attributed to higher MICs, greater stability of the mutation, and better strain fitness (higher growth rate) compared to other 23S mutations that confer macrolide resistance (4, 21).

As a rule, fewer copies of the *rrm* operon increase the likelihood that a mutation in the 23S rRNA will confer macrolide resistance in the organism. Thus, the mutation is seen most commonly in pathogens containing only one or two copies of the *rrm* operon (33). The reason for this is not clear, although it may be that the degree of resistance is linked to the proportion of modified ribosomes and that the mutation must be present in multiple copies in organisms containing more than three copies of *rrm* (5, 25). Among organisms containing three or more copies of *rrm*, macrolide resistance due to a 23S rRNA mutation at the nucleotide equivalent to 2058 has been found in clinical isolates of *Propionibacterium acnes* (three copies of *rrm*) (24) and in laboratory strains of *Streptococcus pneumoniae* (four copies of *rrm*) (29). Our search of the Sanger Center’s incomplete genomic sequence of *B. pertussis* Tohama I strain for sequences homologous to the 23S rRNA gene recognized three occurrences of the 23S gene with nonidentical flanking sequences, supporting the previous report of three copies of the *rrm* operon in this strain (20). The presence of three copies of the 23S gene may explain the rarity of erythromycin resistance in this organism, although it is not known if *B. pertussis* uniformly harbors three copies.

Bacteria can harbor more than one copy of a nucleotide sequence on their chromosome; heterozygosity occurs when different nucleotides occur at a given position among the alternative copies of the sequence. Heterozygosity among *rrm* alleles has been observed in organisms that contain two or more copies of the *rrm* operon, so *B. pertussis* is a candidate in this regard. A mutation in only one of the two copies of the *rrm* operon in clinical isolates of *H. pylori* is sufficient to confer erythromycin resistance, and it appears that the A2058G-equivalent mutation is dominant over the wild-type gene(s) (11, 32). In a study in which four erythromycin-resistant strains of *S. pneumoniae* were generated in the laboratory, three strains contained mutations in at least two of the four copies of *rrm*; while only one strain was homozygous for the mutation (29). In contrast, all erythromycin-resistant isolates of *Propionibacterium* spp., which may contain up to three copies of the *rrm* operon, depending on the species, have been homozygous for the mutation (24).

In the present study, we found evidence for both homozygous and heterozygous *rrm* operons in erythromycin-resistant isolates of *B. pertussis*: two strains, MN2531 and C353, were homozygous for the A2047G mutation, while two others, A228 and C352 were heterozygous. A fifth strain, C310, appears to be a mix of heterozygous and homozygous strains (data not shown). C352 exhibits a novel heterogeneous erythromycin-resistance phenotype that has been described recently (37). A similar phenotype conferred by the equivalent mutation in *H. pylori* was ascribed to either interstrain variation or to heterozygosity of the *rrm* alleles (6, 27). A stably macrolide-resistant derivative of C352, strain C353, appears to be homozygous for the A2047G mutation. These data suggest that the switch to macrolide resistance is due to an increased number of mutant copies of *rrm*, presumably as the result of homologous recombination between *rrm* alleles. Based on our quantitative analyses of PCR-RFLP products and the relative amount of residual A peaks at position 2047 of the DNA sequence of sensitive, resistant, and heterogeneous strains, it appears that the erythromycin-resistant strains of *B. pertussis* examined in this study contained either two or three copies of the A2047G mutation, while the heterogeneous strain contained a single copy of the mutation. These results suggest that the A2047G mutation must be present in multiple copies to confer the homogeneous erythromycin resistance phenotype. An alternative explanation is that strain differences exist that may account for differences in expression of the A2047G mutation. The effect of strain variation and mutation copy number on expression of the erythromycin-resistant phenotype in *B. pertussis* awaits additional studies, including the analysis of in vitro-engineered isogenic strains.

Since the emergence of macrolide-resistant *B. pertussis*, monitoring antimicrobial resistance trends among circulating strains has become a public health priority. Monitoring for erythromycin resistance is important not only to identify and investigate individual treatment failures but also to effectively target prevention and control programs by defining the distribution and frequency of resistant isolates. Critical attention to *B. pertussis* resistance is also prompted by the recent increase in reported cases of pertussis in adolescents and adults; since the pertussis vaccine is not licensed for use in these age groups antimicrobial treatment and prophylaxis remain the primary control and prevention mechanism. However, the low sensitivity of primary culture, the diagnostic standard, and increasing use of more-sensitive molecular methods restrict resistance detection by phenotypic methods. Consequently, our ability to identify resistant *B. pertussis* could be reduced, jeopardizing monitoring programs and underrepresenting the true frequency of resistant isolates and treatment failures. The PCR-RFLP assay described in this study could be useful to rapidly identify erythromycin-resistant *B. pertussis* cultures and suggests an approach for detecting resistance directly from clinical specimens. Additional efforts will be required to validate this method for screening cultures and to evaluate its applicability directly on NP specimens. These results provide a basis for tests that will help clinicians, laboratory scientists, and public health practitioners prepare for the emergence of erythromycin resistance among circulating *B. pertussis* strains. The resistance mechanism described herein is the only mechanism described among resistant isolates to date, but these results do not rule out the potential for emergence of alternative resistance mechanisms. Therefore, aggressive follow-up of potential treatment failures with both phenotypic and genotypic susceptibility assays will be important into the foreseeable future.
REFERENCES