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# Detection of Erythromycin-Resistant Determinants by PCR

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**Erythromycin resistance determinants include Erm methylases, efflux pumps, and inactivating enzymes. To distinguish the different mechanisms of resistance in clinical isolates, PCR primers were designed so that amplification of the partial gene products could be detected in multiplex PCRs. This methodology enables the direct sequencing of amplified PCR products that can be used to compare resistance determinants in clinical strains. Further, this methodology could be useful in surveillance studies of erythromycin-resistant determinants.**

Three different mechanisms of erythromycin resistance have been described (26–28, 54). Target modification is mediated by an rRNA *erm* methylase that alters a site in 23S rRNA common to the binding of macrolides, lincosamides, and streptogramin B antibiotics (26, 28, 54). Enzymes (EreA and EreB) that hydrolyze the lactone ring of the macrocyclic nucleus (2–4, 7, 38, 40, 41) and phosphotransferases (type I [*mphA*] [23, 37, 39] and type II [24]) that inactivate macrolides by introducing a phosphate on the 2′-hydroxyl group of the amino sugar have been reported in members of the family *Enterobacteriaceae* and, recently, in *Staphylococcus aureus* (56). The presence of multicomponent macrolide efflux pumps in staphylococci (*msrA*, *msrB*) (15, 21, 22, 31, 45, 46) and *Neisseria gonorrhoeae* (*mtr*) (17, 42) as well as an efflux system in streptococci (*mefA*, *mefE*) (12, 51, 52) has also been documented.

A set of degenerate primers designed to detect the *erm* classes predominantly found in pathogenic bacteria has been reported (5). However, primers designed to detect other erythromycin resistance determinants have not been described, despite the growing citations of the prevalence of these alternate resistance mechanisms (8, 14, 21, 22, 49, 51, 55). To aid in surveillance of erythromycin resistance determinants, we have developed PCR primers that detect the presence of macrolide efflux (*msrA*/*msrB* or *mefA*/*E*) and drug inactivation (*ereA*, *ereB*, *mphA*). Further, we have also designed primers to distinguish the *ermA*, *ermB*, and *ermC* classes found in pathogenic bacteria.

TABLE 1. Origin and description of reference strains

| Organism | Gene | Location of gene(s) | Reference or source |
|---|---|---|---|
| *S. aureus* RN1389 | *ermA* | Tn*554* in the chromosome | 34 |
| *S. pyogenes* AC1 (pAC1) | *ermB* | Plasmid pAC1 | 13 |
| *S. pyogenes* 02C1064 | *mefA*[a] | Unknown | 12 |
| *E. faecalis* JH2-2 (pJH1) | *ermB* | Plasmid pJH1 | 20 |
| *S. aureus* RN4220 (pE194) | *ermC* | Plasmid pE194 | 19 |
| *S. epidermidis* S1187 | *msrA* | Large plasmid | 46 |
| *S. pneumoniae* 02J1175 | *mefE*[b] | Unknown | 52 |
| *E. coli* BM694 (pAT63) | *ereA* | pBR322 plasmid with cloned insert | 41 |
| *E. coli* BM694 (pAT72) | *ereB* | pUC8 plasmid with cloned insert | 3 |
| *E. coli* BM2506 | *mph* type II | Probably plasmid | 24 |
| *E. coli* BM2570 | *ereB*, *ermBC* | Plasmid pIP1527 | 4 |
| *S. aureus* RN450 (pIP524) | *vga* | Plasmid pIP524 | 15 |
| *E. coli* L441D | *mphA* | Unknown | 37 |

[a] *mefA*, macrolide efflux in *S. pyogenes*.
[b] *mefE*, macrolide efflux in *S. pneumoniae*.

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## MATERIALS AND METHODS

**Strains.** Table 1 lists the origins and descriptions of the reference strains. Table 2 describes the clinical strains and their sources.

**Chemicals.** Brain heart infusion was obtained from Difco Laboratories; brain heart infusion and blood agar plates were purchased from Remel Microbiology Products (Lenexa, Kans.). PCR buffers and DNA polymerase were supplied by Perkin-Elmer (Danbury, Conn.), and all DNA primers were purchased from GenoSys Biotechnologies (The Woodlands, Tex.).

**Design of oligonucleotide primers for *erm* determinants.** Using the multiple sequence alignment program available in BLAST, the rRNA methylases were aligned to reveal the presence of regions with invariant amino acids per *erm* class. Regions of seven to eight amino acids present in all deduced sequences of cloned *erm* genes by class were chosen and evaluated for stem-loop structures and specificity for the *erm* class determinant using programs available in the Genetics Computer Group, Inc. (Madison, Wis.), sequence analysis software programs.

The macrolide efflux determinants *msrA* (46, 47) and *msrB* (32) from *Staphylococcus epidermidis* and *Staphylococcus xylosus*, respectively, were compared with each other and with other efflux proteins using the TFASTA program of the Genetics Computer Group. Primers specific for the second ATP-binding cassette region of *msrA* and the single ATP-binding cassette region of *msrB* and distinguishable from *vga*, a streptogramin A putative efflux determinant (1, 15), were designed.

Primers for *ereA* (41), *ereB* (3), and *mphA* (23, 37) were designed from published sequences to provide specific PCR products of 420, 546, and 837 bp, respectively. For these experiments, the *ereA* primers were 5′-AACACCCTGA ACCCAAGGGACG-3′ and 5′-CTTCACATCCGGATTCGCTCGA-3′; the *ereB* primers were 5′-AGAAATGGAGGTTCATACTTACCA-3′ and 5′-CATA TAATCATCACCAATGGCA-3′; the *mphA* primers were 5′-AACTGTACGC ACTTGC-3′ and 5′-GGTACTCTTCGTTACC-3′. Primers for the *mefA*/*mefE* genes in *Streptococcus pyogenes* and *Streptococcus pneumoniae* were designed

TABLE 2. Clinical strains and their sources

| Organism | Source or reference | Original identification no. | Yr isolated |
|---|---|---|---|
| *S. epidermidis* 01B1034 | Jenssen (25) | 226A | 1985 |
| *S. aureus* 01A1018 | Massachusetts | 68933 | 1989 |
| *E. faecalis* 03A1069 | Missouri (CDC)[a] | HIP2025 | 1994 |
| *S. pyogenes* 02C1076 | Ireland | DC680 | 1994 |
| *S. pyogenes* 02C1086 | Indiana | V985100 | 1994 |
| *S. pneumoniae* 02J1126 | California | M28095 | 1994 |
| *S. pneumoniae* 02J1092 | Texas (CDC) | 2033228 | 1994 |

[a] CDC, Centers for Disease Control and Prevention, Atlanta, Ga.

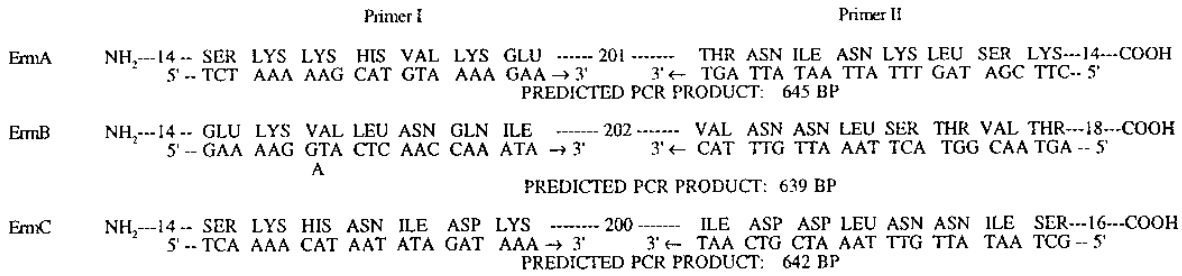


FIG. 1. Sequences and primers to detect *ermA*, *ermB*, and *ermC*. The corresponding codons and nucleotide sequences of oligonucleotide primers for *ermA*, *ermB*, and *ermC* are shown. The numbers of amino acids between the different primer regions and the amino and carboxy termini are indicated for each primer set.

after comparison of the two sequences (12, 52); the primers were 5′-AGTATC ATTAATCACTAGTGC-3′ and 5′-TTCTTCTGGTACTAAAAGTGG-3′.

**Amplification protocol.** Genomic DNA was isolated from *Escherichia coli* strains as described in reference 6. DNA recovery from the other species required modifications (51) to ensure adequate cell breakage.

The PCR mix was as recommended by Perkin-Elmer, with a magnesium concentration of 2 mM being preferred for the *ermA*/*ermB*/*ermC*/*msr* primer set (primer mix I) and 4 mM for the *ereA*/*ereB*/*mphA*/*mef* primer set (primer mix II). When the PCRs were done separately for each erythromycin-resistant determinant, 4 mM magnesium was used for the *ermA*, *msr*, *ereA*, *ereB*, *mphA*, and *mef* primer sets and 2 mM was used for the *ermB*, *ermC*, and consensus *erm* primer sets. PCR conditions were as described in reference 51; electrophoresis on 1% agarose gels in 40 mM Tris acetate–2 mM EDTA buffer was used to distinguish PCR products. The size of each PCR product was estimated using standard molecular weight markers (1-kb DNA ladder from Gibco BRL, Gaithersburg, Md.).

## RESULTS

**Amplification of *erm* and *msr* genes.** Oligonucleotide pairs were designed to hybridize to either *ermA*, *ermB* (including *ermAM* and *ermBP*), or *ermC* sequences from pathogenic species. By comparing published sequences of *erm* genes from different pathogenic species and strains, a minimum of degeneracy was necessary in the design of the *erm* specific primers, allowing for annealing temperatures to be high. Figure 1 details the respective amino acids and nucleotide codons present in the different *erm* classes: *ermA*, *ermB*, and *ermC*. Note that only one deduced sequence of *ermA* was available for use (34), while seven deduced sequences were used for *ermB* (9, 11, 16, 18, 44, 50, 53), and five sequences were evaluated for *ermC* (10, 19, 25, 33, 43).

Because the expected PCR product from any of the *erm* primers (~640 bp) (Fig. 1) can be distinguished from the expected PCR product from *msrA*/*msrB* primers (~400 bp) (Fig. 2), PCR using the four primer sets was performed simultaneously on genomic DNA samples from each of the reference strains (Table 1). The reference strains contain either a single copy (*ermA*), multiple copies (*ermB*, *ermC*, *msrA*, *ereA*, *ereB*, or *vga*), or an unknown number of copies (*mphA*, *mefA*, *mefE*) of the specified antibiotic-resistant determinant. If a strain was shown to contain an *erm* gene, it was subjected to a secondary assay with individual primer sets to resolve the determinant as *ermA*, *ermB*, or *ermC*. Because of the specificity of the primers and the reaction conditions, nonspecific bands were rarely noted with any species or strain tested. Another measure of integrity of the reaction mix and primer sets is inherent in the DNA sample from *S. aureus* RN1389, a strain that contains a single chromosomal copy of *ermA*. Each of the reference strains gave the expected results: *ermC* was found in *S. aureus* RN4220 (pE194), and *ermB* was detected in *S. pyogenes* AC1 (pAC1), *Enterococcus faecalis* JH2-2 (pJH1), and *E. coli* BM2570 (Fig. 3) (secondary assay data not shown).

The *msrA*/*msrB* primer set produced a distinguishable PCR product with *S. epidermidis* S1187, the reference strain for *msrA*, without producing a false positive with *S. aureus* RN450 (pIP524), a strain that carries a plasmid encoding *vga*, a putative efflux protein for streptogramin A closely related to *msrA*/*msrB* (Fig. 2). The *vga* gene sequence (1) differed by seven nucleotides (and two amino acids) within the E1 primer and by seven nucleotides (and two amino acids) within the E2 primer from *msrA*/*B* (Fig. 2).

**Amplification of *ereA*, *ereB*, *mphA*, and *mef* determinants.** EreA and EreB are esterases found in enteric strains highly resistant to erythromycin (2–4, 7, 38, 40, 41). Two different types of macrolide phosphotransferases have been distinguished based on enzymatic characterization (24, 39). However, the structural determinant for only type I, *mphA* (37) or *mphK* (23), has been determined. Recently, a macrolide efflux pump was described in *S. pyogenes* (12) and *S. pneumoniae* (51, 52). The pump, called *mefA* in *S. pyogenes* and *mefE* in *S. pneumoniae*, appears to belong to the major facilitator family (29, 36, 52) as it requires an active proton motive force (12, 51, 52). The two genes are ~90% identical (12, 52), and this form of macrolide resistance is prominent in recent clinical isolates (51).

PCRs using the primer sets designed for *ereA*, *ereB*, *mphA*, and *mef* could be performed with a mix containing the four primer sets. The different product sizes (420 bp for *ereA*, 546 bp for *ereB*, 837 bp for *mphA*, and 348 bp for *mefA*/*E*) are easily distinguishable on a 1% agarose gel. Figure 3 shows that when primer mix II was used to amplify DNA from strains with defined erythromycin-resistant determinants, an *ereA* product was seen with *E. coli* BM694 (pAT63); an *ereB* product was

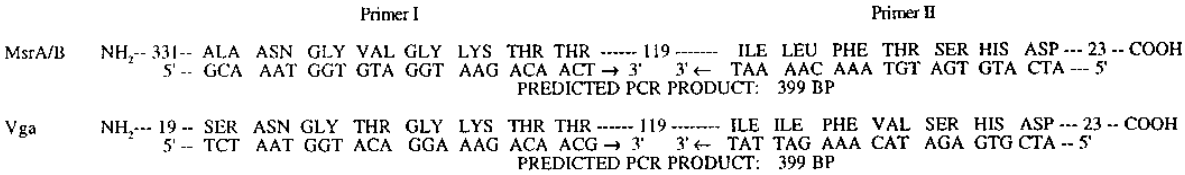


FIG. 2. Sequences and primers to detect *msrA*/*msrB* from *vga*. The corresponding codons and nucleotide sequence of oligonucleotide primers for *msrA*/*msrB* and *vga* are shown. The numbers of amino acids between the different primer regions and the amino and carboxy termini are indicated for each primer set.

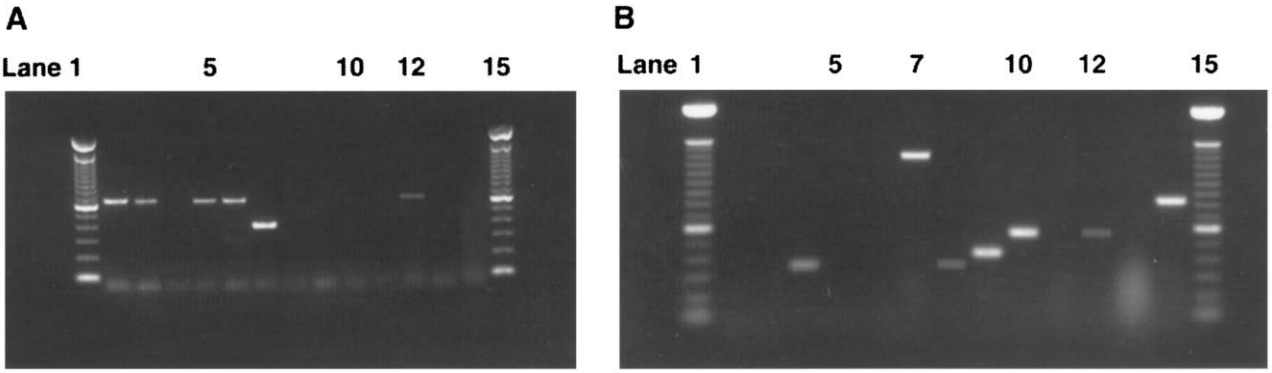


FIG. 3. PCR analysis of erythromycin-resistant determinants with reference strains. (A) PCR products observed using primer mix I (*ermA*, *ermB*, *ermC*, *msrA*/*msrB*); (B) PCR products observed using primer mix II (*ereA*, *ereB*, *mphA*, *mef*). Source of DNA for each lane: lanes 1 and 15, 1-kb DNA ladder; lane 2, DNA from *S. aureus* RN1389 (*ermA*); lane 3, *S. pyogenes* AC1 (pAC1) (*ermB*); lane 4, *S. pyogenes* 02C1064 (*mefA*); lane 5, *E. faecalis* JH2-2 (pJH1) (*ermB*); lane 6, *S. aureus* RN4220 (pE194) (*ermC*); lane 7, *S. epidermidis* S1187 (*msrA*); lane 8, *S. pneumoniae* O2J1175 (*mefE*); lane 9, *E. coli* BM694 (pAT63) (*ereA*); lane 10, *E. coli* BM694 (pAT72) (*ereB*); lane 11, *E. coli* BM2506 (*mph* type II); lane 12, *E. coli* BM2570 (*ereB*, *ermBC*); lane 13, *S. aureus* RN450 (pI524) (*vga*); lane 14, *E. coli* L441D (*mphA*). See Table 1 for origin of reference strains.

detected with *E. coli* BM694 (pAT72) and *E. coli* BM2570; an *mphA* product was observed with *E. coli* L441D and, notably, not with *E. coli* BM2506 (contains type II macrolide-2′-phosphotransferase); and an *mefA*/*E* product was seen with *S. pyogenes* 02C1064 and *S. pneumoniae* 02J1175. Interestingly, we observed a 1.2-kb product with DNA from *S. epidermidis* S1187, the reference strain for *msrA* (46). When the PCRs were primed separately, the product was found to be specific to the *mphA* primers (data not shown). This result supports our observation that this strain inactivates erythromycin (57) as well as effluxes macrolides (46). No products were seen in strains known to contain only Erm methylases.

**Detection of erythromycin-resistant genes in clinical gram-positive isolates.** The primer sets described in this report were used to amplify genomic DNA from representative isolates of erythromycin-resistant *S. epidermidis*, *S. aureus*, *E. faecalis*, *S. pyogenes*, and *S. pneumoniae* (Fig. 4). Erythromycin resistance in the *S. epidermidis* clinical strain O1B1034 appears to be both efflux mediated and due to inactivation as evidenced by our previous results (57) and confirmed in the PCR analysis. An *msrA*-specific PCR product (~400 bp) as well as an unexpected 1.2-kb product was seen. Like the 1.2-kb product observed for the reference strain *S. epidermidis* S1187 (Fig. 3 and Table 1), this band was present only in *S. epidermidis* 01B1034 when DNA was amplified using the *mphA* primer set (data not shown).

The *S. aureus* and the *E. faecalis* strains have an *erm* gene and no other erythromycin-resistant determinants; secondary evaluation of these strains using separate primer sets for the *erm* genes revealed that *S. aureus* 01A1018 had *ermA* and *E. faecalis* 03A1069 had *ermB* (data not shown). PCR analyses with two *S. pyogenes* and two *S. pneumoniae* strains revealed that one isolate of each species contains a prototypical *ermB* gene while the other isolate has the novel *mefA*/*E* gene.

## DISCUSSION

This paper describes PCR primers that can be used to survey erythromycin-resistant mechanisms present in clinical isolates. Utilization of the multiplex PCR technology in the clinical

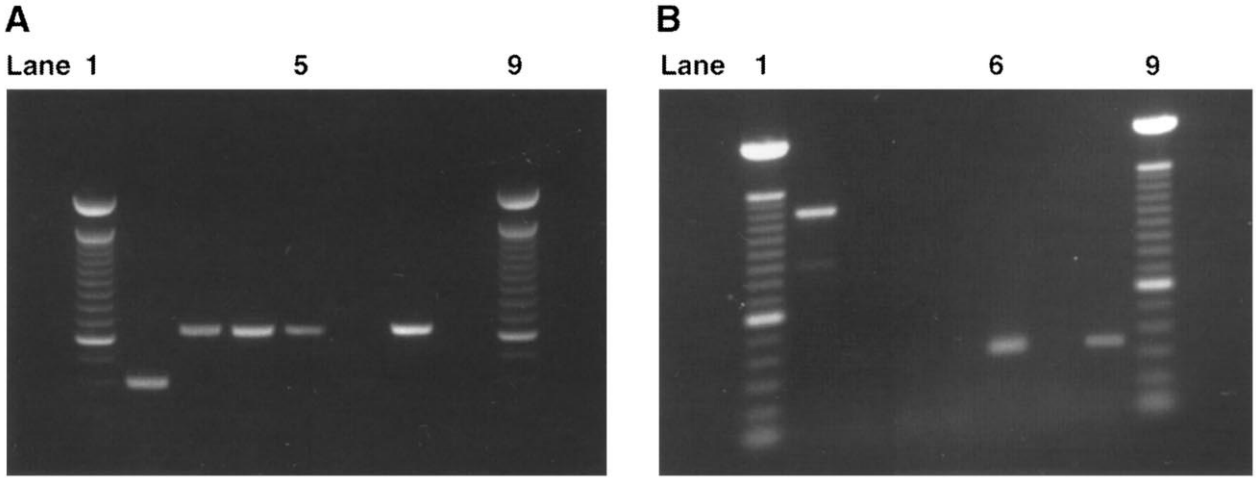


FIG. 4. PCR analysis of erythromycin-resistant determinants with clinical strains. (A) PCR products observed using primer mix I (*ermA*, *ermB*, *ermC*, *msrA*/*msrB*); (B) PCR products observed using primer mix II (*ereA*, *ereB*, *mphA*, *mefA*/*E*). Source of DNA for each lane: lanes 1 and 9, 1-kb DNA ladder; lane 2, *S. epidermidis* 01B1034; lane 3, *S. aureus* 01A1018; lane 4, *E. faecalis* 03A1069; lane 5, *S. pyogenes* 02C1076; lane 6, *S. pyogenes* 02C1086; lane 7, *S. pneumoniae* 02J1126; lane 8, *S. pneumoniae* 02J1092. See Table 2 for origin of clinical strains.

laboratory in combination with the recommended National Committee for Clinical Laboratory Standards guidelines (35) would better enable physicians to prescribe appropriate antibiotic therapy, leading to therapeutic success, more prudent antibiotic usage, and conditions less conducive to resistance selection.

From a discovery perspective, this methodology permits the direct sequencing of amplified PCR products to yield partial gene sequences for comparison from many different bacterial species. The ability to combine different primer sets in a multiplex reaction not only allows the detection of erythromycin-resistant determinants but provides a ready means for distinguishing the nature of the resistance determinant. Although the primers were designed to be specific, the nature of the cycling conditions favors sufficient promiscuity to detect closely related elements. An example of this became evident when we detected a putative *mphA*-like determinant in *S. epidermidis* S1187 (Fig. 3 and Table 1) and 01B1034 (Fig. 4 and Table 2).

The PCR multiplex methodology to detect resistance determinants can be made more powerful when combined with hybridization and sequencing studies. The success of the PCR approach alone may, however, follow reports that have recently surfaced describing PCR protocols that are as reliable as pulsed-field gel electrophoresis for strain serotyping (30) or that are sufficient to diagnose species-specific determinants (48).

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