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Identification of a Novel Keyhole Phenotype in Double-Disk Diffusion Assays of Clindamycin-Resistant Erythromycin-Sensitive Strains of *Streptococcus agalactiae*

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Our objective was to characterize 46 unique, erythromycin-sensitive, and clindamycin-resistant *Streptococcus agalactiae* strains from S. Korea that displayed a novel phenotype in double-disk diffusion assay. We used polymerase chain reaction to determine presence of erythromycin and clindamycin resistance genes, disc diffusion assays to determine resistance phenotype, and microbroth dilution to determine minimal inhibitory concentration. We detected a novel phenotype in the double-disk diffusion assay for inducible resistance among 46 *S. agalactiae* strains that were both erythromycin sensitive and clindamycin resistant. Thirty-two strains with the novel phenotype tested positive for *erm*(B) by DNA–DNA hybridization; sequencing of the *erm*(B) gene revealed mutations in the ribosomal binding site region in the *erm*(B) open reading frame, which is consistent with a lack of erythromycin resistance phenotype. Although identified from patients at multiple hospitals, genotyping suggested that the strains are closely related. The new phenotype shows increased sensitivity to clindamycin in the presence of erythromycin.

Introduction

Streptococcus agalactiae (*S. agalactiae*) is a common cause of severe infections in neonates, and causes bacteremia and endocarditis in immunocompromised populations.[5] *S. agalactiae* remains sensitive to treatment with penicillin; erythromycin or clindamycin is the recommended alternative for patients who are β-lactam intolerant.[4] Resistance to erythromycin and clindamycin has increased during the last decade and is mainly attributed to the erythromycin-conferring methylase genes (*erm*). The *erm* genes code for ribosomal methylase and confer cross resistance to clindamycin either through constitutive or inducible resistance mechanisms.[1] Thus, resistance to erythromycin and clindamycin often occurs together. Resistance to erythromycin alone can be conferred by the efflux gene *mef*, and resistance to clindamycin alone can be conferred by the *lnu*(B) gene that expresses a specific nucleotidyl transferase.[5]

As part of a study of antibiotic resistance determinants in *S. agalactiae* collected in South Korea in 2006–2007, we identified 46 strains that displayed high resistance to clindamycin, yet were sensitive to erythromycin. We performed the double-disk diffusion testing for inducible resistance and observed a novel phenotype characterized by a channel of sensitivity to clindamycin in the presence of erythromycin. The characteristics of this novel phenotype with increased sensitivity to clindamycin in the presence of erythromycin are reported here.

Materials and Methods

Study populations and S. agalactiae *isolation*

The study collection included 197 colonizing isolates collected from 140 asymptomatic pregnant women (35–37 weeks of gestation) and 233 clinical isolates collected from 231 patients with *S. agalactiae* infection. One unique isolate per individual was selected for further study ($n = 371$). Colonizing isolates were collected from pregnant women receiving prenatal care at four hospitals, Eulji Hospitals in Seoul and Daejeon, Cheil Hospital in Seoul, and Motae

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Women's Hospital in Daejeon, South Korea, between 2006 and 2007. Invasive *S. agalactiae* strains were isolated from specimens sent for microorganism culture from hospitals and clinics throughout the country between January 2006 and March 2007.[7] Written informed consent permitting use of collected specimens and medical records for research purposes was obtained from each patient. Mucus or discharge was collected from patients with infection, and rectal swabs and urine from pregnant women. All isolates were cultured by using a selective medium (Todd-Hewitt broth supplemented with gentamicin [8 mg/ml] and nalidixic acid [15 mg/ml], or with colistin [10 mg/ml] and nalidixic acid [15 mg/ml]) to inhibit growth of bacteria other than *S. agalactiae*. The identity of *S. agalactiae* strains was confirmed by catalase test followed by a latex agglutination assay (Streptex; Murex Biotech Ltd.). Antibiotic susceptibility for *S. agalactiae* was tested by two methods: (1) minimal inhibitory concentration (MIC) was determined by broth dilution assays; (2) disc diffusion was used to study inducible resistance to clindamycin in the presence of erythromycin and to identify the novel keyhole phenotype. Strains with MIC $\leq$0.25 μg/ml were designated as erythromycin/clindamycin susceptible, whereas MIC $\geq$1 μg/ml were designated as erythromycin/clindamycin resistant. Follow-up disc diffusion testing used commercial antibiotic discs (Oxoid) and Mueller–Hinton with 5% (v/v) sheep blood with erythromycin and clindamycin discs and were interpreted using published Clinical and Laboratory Standards Institute standards. If an isolate was either erythromycin-resistant/clindamycin-susceptible or erythromycin-susceptible/clindamycin-resistant, we performed a disk induction test by placing clindamycin (2 μg) and erythromycin (15 μg) disks 12 mm apart following CLSI recommendations.[2] The plates were examined after incubation at 35°C, 5% CO_2 for 20–24 hours; an isolate with a clindamycin disk diffusion zone blunted on the side closest to erythromycin (D test) was considered to be inducible. Strains demonstrating the new phenotype with the clear channel of inhibition between the clindamycin and erythromycin discs were re-tested with different lots of antibiotic discs and media to rule out nonspecific effects. Serotype was determined using the *S. agalactiae* kit (Essum®).

Detection of resistance genes

Purified *S. agalactiae* genomic DNA was arrayed on Vivid Gene Array slides (Pall), as previously described.[9] Polymerase chain reaction (PCR) amplification of antibiotic resistance genes was performed using *S. agalactiae* overnight cultures, 50 pmol of each primer, and 45 μl of Accuprime super mix II (Invitrogen). Primers to detect *erm*(B), *lnu*(B), *erm*(A), and *mef*(A) were described previously and used to prepare labeled probes.[7] The presence of antibiotic resistance genes was determined using high-throughput DNA–DNA hybridization as previously described,[2] and PCR was performed when hybridization results were inconclusive.

The *lnu*(B) gene was identified using DNA–DNA hybridization with a probe targeting a 944 bp region of the *lnu*(B) gene, and hybridization conditions would have detected signal in the absence of a perfect probe–DNA match. For samples that were *lnu*(B) negative by DNA–DNA hybridization, PCR amplifications were attempted using both colony PCR and purified genomic DNA as template in replicates. If neither of the PCR gave expected size products, the strains were designated as *lnu*(B) negative.

A 639 bp fragment of *ermB* gene was amplified using 5′-GAA AAG GTA CTC AAC CAA ATA-3′ and 5′-AGT AAC GGT ACT TAA ATT GTT TAC-3′ (reverse). Primers designed to PCR amplify the entire *erm*(B) gene in the novel phenotype strains were not successful; hence, a 1,000 bp region encompassing the *erm* and adjacent genetic region in *S. agalactiae* was amplified using primers GTATTGTCCGAGAGTGATTGG TC and AACTTACCCGCCATACCACAG from previously published literature on *erm*-positive *S. agalactiae* transposon Tn3872.[8] PCR conditions used included denaturation at 94°C for 2 minutes, followed by 30 cycles at 94°C for 30 seconds of denaturation, 55°C for 1 minute, and extension at 72°C for 2 minutes. PCR products were purified and sequenced at from 5′ and 3′ ends at the DNA Sequencing core, University of Michigan.

Results

Figure 1 shows an example of the novel phenotype in double-disk diffusion assay, induced clindamycin sensitivity

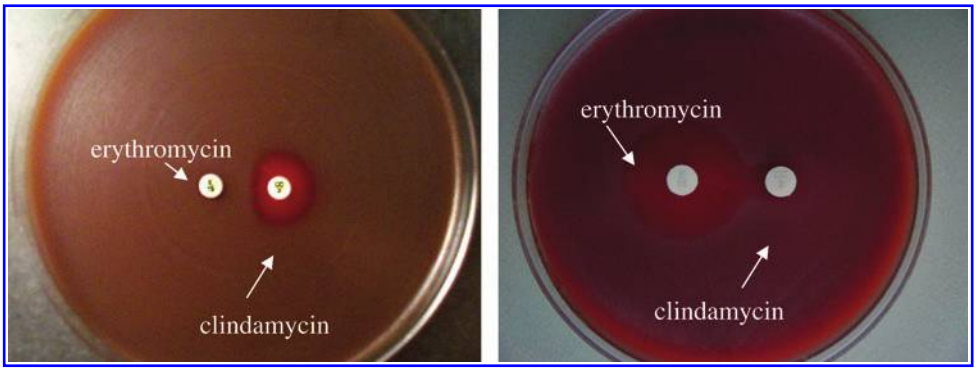

FIG. 1. Erythromycin and clindamycin double-disk diffusion assays of *Streptococcus agalactiae* from South Korea. Strains were grown to 0.5% McFarland and inoculated on to Mueller–Hinton with 5% (v/v) sheep blood and allowed to grow at 35°C for 16–18 hours. The left panel shows erythromycin-induced clindamycin resistance seen as a blunted "D" inhibition zone. The right panel shows the novel keyhole phenotype seen as an enhancement of the zone of inhibition between erythromycin and clindamycin discs in erythromycin-sensitive and clindamycin-resistant *S. agalactiae* strains. Color images available online at www.liebertonline.com/mdr.

in a keyhole phenotype. The 46 strains with the novel phenotype were isolated from 28 patients with disease caused by *S. agalactiae* and 18 pregnant patients undergoing screening for *S. agalactiae.* The keyhole phenotype strains were isolated from urine ($n = 25$), rectal swabs ($n = 7$), vaginal swabs ($n = 4$), cervical swabs ($n = 3$), pus ($n = 2$), wound ($n = 1$), and 4 from other sites. In seven individuals the keyhole phenotype colonized multiple sites (only one strain per individual was counted toward the 46 strains exhibiting the novel phenotype). Forty-three of these strains (95%) were serotype III; the three remaining strains were serotype Ib and Ia. To determine the relatedness of the strains with the phenotype, we sequenced the *S. agalactiae adpH* gene to identify polymorphisms. The variable *adpH* gene has a high number of mutations that are used to differentiate between strains using multi-locus sequence typing. We did not find any sequence differences in the *adpH* genes from the 46 *S. agalactiae* strains exhibiting the novel phenotype, suggesting that the strains are closely related genetically.

To identify the cause of clindamycin and erythromycin resistance, we screened for the presence of several genes. The *lnu*(B) gene is associated with clindamycin resistance, and 42/46 of the erythromycin-susceptible, clindamycin-resistant strains with the keyhole phenotype had *lnu*(B) by PCR (Table 1). One isolate was *lnu*(B) positive by hybridization but tested *lnu*(B) negative by PCR (data not shown). The three other strains with the keyhole phenotype were *lnu*(B) negative (Table 1). Erythromycin and clindamycin resistance-conferring genes *erm*(A) and *mef*(A) were not found in any of the strains exhibiting the novel phenotype.

Thirty-two strains displaying the keyhole phenotype were *erm*(B) positive by DNA–DNA hybridization and PCR (Table 1), yet remained phenotypically erythromycin sensitive. PCR amplification of the *erm*(B) gene revealed a point mutation in the leader sequence (AGGAGT to ATTAGA) and three point mutations in the coding region of the *erm*(B) gene; at 222 bp (C to T), 224 bp (T to C), and 299 bp (A to G), respectively. These mutations were absent in 30 erythromycin and clindamycin-resistant strains that did not show the keyhole phenotype (data not shown). We were unable to amplify a region of 57 bp from the 3′ coding region of *erm*(B) as well as the region immediately downstream of the *erm*(B) gene in the strains exhibiting the novel phenotype.

MIC values for clindamycin resistance were >8 μg/ml for all strains with the keyhole phenotype (data not shown). We selected five strains to determine the effect of erythromycin on MIC of clindamycin; all exhibited the keyhole phenotype and were *erm*(B) and *lnu*(B) positive. However, when the strains were inoculated with erythromycin and clindamycin, the results were mixed; three out of the five strains tested had decreased MIC values for clindamycin when inoculated with low concentrations of erythromycin; the clindamycin MIC values for two other strains remained unchanged (data not shown).

Table 1. Prevalence of Antibiotic Resistance Genes in 46 Erythromycin-Sensitive, Clindamycin-Resistant *Streptococcus agalactiae* Strains Exhibiting a Keyhole Phenotype in Double-Disk Diffusion Assay

Keyhole phenotype strain characteristics	*Number of strains (%)*
Site of isolation	
Urine	25 (54%)
Rectum	7 (15.2)
Cervix	3 (6.5%)
Vagina	4 (9%)
Other[a]	7 (15%)
Serotype	
III	43 (94%)
Ia	1 (2%)
Ib	2 (4%)
Resistance-conferring genes	
ErmB	32 (70%)
LnuB	43 (94%)
ErmA	0 (0%)
MefA	0 (0%)

[a]Other sites include wound and pus, and sites of collection for some strain were not available.

Discussion

We characterized 46 *S. agalactiae* with a unique keyhole phenotype that is best described as inducible clindamycin *sensitivity.* The 46 clindamycin-resistant and erythromycin-sensitive strains with the phenotype were identified from both clinical and colonizing isolates in South Korea between 2006 and 2007 and did not cluster by hospital. However, genotyping suggested that the strains were closely related. All strains were more susceptible to clindamycin in the presence of erythromycin in the double-disk diffusion assay than suggested from MICs or from a single-disc diffusion assay. Almost all (94%) strains carried the *lnu*B gene, which codes for clindamycin resistance, and 70% carried a non-functional *erm*B, which is consistent with erythromycin-susceptible phenotype of these strains. The mechanistic basis of the channel of zone inhibition between the erythromycin and the clindamycin disks in strains exhibiting the keyhole phenotype in *S. agalactiae* strains is not known. Since the novel phenotype was observed in both *lnu*(B)-positive and *lnu*(B)-negative strains as well as *erm*(B)-positive and *erm*(B)-negative strains, we postulate that the mechanism underlying the novel phenotype is either independent of the *erm*(B) and *lnu*(B) genes or is associated with *erm* and *lnu* variants that were not detected by probes used for DNA–DNA hybridization or primers used for PCR methods in this study.

Sequence analysis of the *erm*(B) region in keyhole phenotype strains that were positive for *erm*(B) revealed point mutations in both the Shine Dalgarno ribosome binding sequences and *erm*(B) coding regions. Mutations in the Shine and Dalgarno sites are associated with reduced translation and may be responsible for the erythromycin sensitivity of these *erm*(B)-containing strains. Two point mutations within the *erm*(B) coding region were also detected in all strains exhibiting the novel phenotype that were positive for *erm*(B). One mutation codes for an amino acid substitution from Asp174Thr on the *erm*(B) coding region; Asp174 has been implicated to be important in the binding of the substrate, S-adenosine methionine to *erm*(B) in *Clostridium*[3]; however, their functional significance in strains with the keyhole phenotype is not known. The second mutation, a deletion in the 3′ end of the *erm*(B) coding region, would also serve to explain the erythromycin sensitivity of these strains with the

keyhole phenotype. The presence of identical mutations on all *erm*(B)-positive strains exhibiting the keyhole phenotype implies that the *erm*(B) gene may have originated from a common ancestor or have mutated due to similar selective pressures to lose function. A more detailed molecular typing and phylogenetic analysis of these strains is planned.

In the laboratory, constitutively resistant mutants can be selected at high frequencies from inducible resistant strains in the presence of clindamycin; in a clinical setting, this implies that there is a risk for selecting clindamycin-resistant strains if infections are treated with clindamycin.[6] We do not know the implications of the keyhole phenotype *in vivo*, but it is possible that these strains would respond to erythromycin therapy.

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Disclosure Statement

The authors have no conflicts of interest to declare.

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