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Antimicrobial Susceptibilities of Invasive Pediatric *Abiotrophia* and *Granulicatella* Isolates

Xiaotian Zheng,[1]* Alexandra F. Freeman,[1] Jay Villafranca,[1] Dee Shortridge,[2] Jill Beyer,[2] William Kabat,[1] Karen Dembkowski,[1] and Stanford T. Shulman[1]

Children's Memorial Hospital/The Feinberg School of Medicine, Northwestern University, Chicago,[1] *and Abbott Laboratories, Abbott Park,*[2] *Illinois*

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***Abiotrophia* and *Granulicatella* species have been associated with various infections. Antimicrobial susceptibility data for these nutritionally variant streptococcus-like organisms, especially for pediatric isolates, are very limited. Little is known about the genetic bases of their resistance mechanisms. We report the results of identification to bacterial species level, antimicrobial susceptibility testing, macrolide resistance testing, and detection of genes encoding that resistance for a collection of 15 pediatric clinical isolates from normally sterile sites. Our results indicate that the prevalence of beta-lactam and macrolide resistance is high and that both *erm* and *mef* are found in these isolates.**

Originally known as nutritionally variant streptococci, *Abiotrophia* and *Granulicatella* species are part of the normal human oral and intestinal flora. They were originally described in 1961 by Frenkel and Hirsch (8). In 1989, based on DNA-DNA hybridization studies, these organisms were classified into two groups: *Streptococcus defectivus* and *Streptococcus adjacens* (3). Based on 16S rRNA gene sequences and phenotypic characteristics, the two species subsequently were transferred to the new genus *Abiotrophia* in 1995 (10). Since then, three new species, *A. elegans*, *A. balaenopterae*, and "*A. para-adiacens*," have been added. Most recently, these species were proposed to be reclassified into two genera, *Abiotrophia* and *Granulicatella* (6, 7).

These bacteria have been associated with various infections including bloodstream infection and infective endocarditis (5, 16, 20). Other reported infections include otitis media, brain abscess, and septic arthritis (2, 9). Because of their unique growth requirements, relatively uncommon recovery from clinical specimens, and the lack of standardized testing methodology and interpretation, limited antimicrobial susceptibility data for these organisms, especially for pediatric isolates, are available. Little is known about the genetic basis of their resistance mechanisms. Here we report results of antimicrobial susceptibility testing and the detection of genes encoding resistance to macrolides for a collection of 15 pediatric clinical isolates from normally sterile sites collected at the Children's Memorial Hospital during the last 4 years.

Laboratory testing, results, and discussion. The 15 clinical isolates from normally sterile pediatric sites (12 from blood, two from cerebrospinal fluid [CSF], and one from peritoneal fluid) had been stored at −70°C before the study. All isolates grew on chocolate agar plates but not on 5% sheep blood agar plates (BBL). In addition, they all grew well as satellite colonies around *Staphylococcus aureus* colonies on 5% blood agar plates. Species-level identification was performed with conventional biochemical reaction tests and the API 20 Strep identification system (bioMerieux, Inc., Hazelwood, Mo.). The key biochemical reactions for species-level identification included hydrolysis of arginine and acid production from trehalose and sucrose (6).

Antimicrobial susceptibility testing was performed by standard broth microdilution methods (Microtech Medical Systems, Inc., Aurora, Colo.) with pyridoxal-supplemented medium (0.001%). To evaluate the MIC over a broader range of concentrations, erythromycin and clindamycin were tested with user-prepared microdilution plates that included 5% lysed horse blood and pyridoxal supplement (14, 19). Four isolates did not grow by 24 h in broth and were tested by Etest strips (AB Biodisk, Solna, Sweden) for these two drugs. Interpretation of antimicrobial susceptibility testing results for most drugs was based on the NCCLS interpretive criteria for the viridans group described in the table for *Streptococcus* spp. other than *Streptococcus pneumoniae* (14). Since there are no NCCLS breakpoints for ciprofloxacin and rifampin against these organisms, a MIC of ≥4 μg/ml was used as the level for ciprofloxacin resistance (4), and interpretation standards for *S. pneumoniae* were used for rifampin.

To assess the mechanism of macrolide resistance of these organisms, PCR was performed using primers specific for the known streptococcal resistance determinants *mef*(A), *erm*(A), *erm*(B), and *tet*(M) as previously described (1, 17, 18). Briefly, crude DNA lysate of each strain was prepared by boiling cell suspension for 10 min; following centrifugation to pellet cell debris, 1 μl of supernatant was used in the amplification reaction. PCRs were performed in a 25-μl volume with PCR Supermix as recommended by the manufacturer (Gibco BRL, Rockville, Md.) on a Gene Amp System 9700 (Applied Biosystems Inc., Foster City, Calif.). Primer pairs for resistance determinants examined along with the region of the gene amplified, annealing temperature, and primer reference are shown in Table 1. The thermal cycling profile was 30 cycles of amplification: denaturation at 94°C for 30 s, annealing at 45 to 52°C (temperatures for each primer pair are shown in Table 1) for 30 s, and extension at 72°C for 45 s. PCR products were

* Corresponding author. Mailing address: Children's Memorial Hospital, 2300 Children's Plaza, Box 53, Chicago, IL 60614. Phone: (773) 880-6910. Fax: (773) 880-4687. E-mail: x-zheng@northwestern.edu.

TABLE 1. PCR primer information

Gene	Primer sequence, 5′–3′	Region amplified (bp)	Annealing temp (°C)	Reference
erm(A)	Forward: AGAACAATCAATACAGAGTC Reverse: TGAACCAGAAAAACCCTAAA	4756–5282	45	Shortridge et al. (17)
erm(B)	Forward: TCAACCAAATAATAAAACAA Reverse: AATCCTTCTTCAACAATCA	974–1311	45	Shortridge et al. (17)
mef(A)	Forward: ATGCAGACCAAAAGCCACAT Reverse: GCCATAGACAAGACCATCGC	369–695	52	Shortridge et al. (18)
tet(M)	Forward: CATGTTGATGCGGGAAAAAC Reverse: CACTTCCGTGATAAACAGGG	30–661	50	Almer et al. (1)

detected and identified by electrophoresis through 2% agarose gels run at 100 V, followed by ethidium bromide staining and comparison to molecular weight standards (100-bp ladder; Gibco BRL). The primers were chosen with Oligo 5.0 (NBI Software, Plymouth, Minn.) from sequences deposited in GenBank (Bethesda, Md.). A positive control with chromosomal DNA previously shown to have the gene being tested and a negative amplification control were included in each PCR run.

DNA sequencing for the determination of *tet*(M) was conducted as previously described (1). Briefly, the amplification product was sequenced using the Big Dye sequencing kit (Applied Biosystems Inc.). Sequencing reaction mixtures were purified with an Auto-Seq G-50 column (Amersham Pharmacia Biotech, Piscataway, N.J.), and reactions were run on an ABI 377 automated sequencer. The DNA sequence of amplified products from the strain in question was compared to the public sequence of the target gene (accession no. X04388) and found to be identical to the wild-type sequence. The region amplified did not include upstream regulatory regions; therefore, the reason for the lack of expression was not determined.

The API 20 Strep identification system gave the following identification codes: 0140000 (three isolates), 0540000 (three isolates), 0350451 (two isolates), 0140100 (two isolates), 0350011 (two isolates), 0350411, and 0140020. One isolate became nonviable, and the API 20 Strep test was not done. The system called the isolates tested either *Abiotrophia adiacens* or *Abiotrophia defectiva*, and most identifications were low discriminatory. The species-level identification in this study was based on conventional tests (Table 2). The 15 isolates included six of *A. defectiva*, six of *G. adiacens*, and three of *G. elegans*. Of these, two were susceptible (MIC, ≤0.12 μg/ml), 10 were in the intermediate category (MIC, 0.25 to 2 μg/ml), and three were resistant to penicillin, with MICs being ≥4 μg/ml (Table 3). For 13 of the 15 isolates the cefuroxime MIC was >2 μg/ml, 9 of 15 isolates were resistant to ceftriaxone (MIC of >2 μg/ml), 2 of 15 were resistant to tetracycline (MIC of >8 μg/ml), two were intermediate and 13 were susceptible to chloramphenicol, for one the trimethoprim-sulfamethoxazole MICs were 2 and 38 μg/ml, respectively, and for 14 isolates the MICs were <0.5 and 9.5 μg/ml, respectively. Given the clinical utility of rifampin, susceptibility testing was performed by Etest. For all the isolates tested, the MICs of rifampin were low (≤0.012 μg/ml). In addition, all isolates were susceptible to vancomycin (MIC, <1 μg/ml).

Eight of 15 isolates (53%) were found to be macrolide resistant, with erythromycin MICs ranging from 2 to >64 μg/ml (Table 4). Three erythromycin-resistant isolates were also clindamycin resistant.

As shown in Table 5, although there are a limited number of isolates in the present study, the resistance rates in the present study appear higher than those in the recently published data by Tuohy et al. (19) and Murray et al. (13). This is seen in various drug classes including beta-lactams, macrolides, clindamycin, and tetracycline. Possible reasons for the higher resistance rates are that the specimens in the present study were from pediatric patients and that children may possess flora that have been exposed to more antibiotics. A more recent article from Taiwan reported a very high prevalence of resistance to

TABLE 2. Clinical diagnosis and laboratory identification of the 15 cases[a]

Isolate no.	API code and ID	Age (yr)/sex	Diagnosis	Source	Identification
1	0140000, *A. defectiva*	1.5/F	Medulloblastoma; ventriculoperitoneal shunt	CSF, EVD	*G. adiacens*
2	0140000, *A. adiacens*	3/M	Leukemia	Blood	*G. elegans*
3	0350451, *A. defectiva*	5/M	Sickle cell disease	Blood	*A. defectiva*
4	0540000, *A. adiacens*	9/F	Chronic pancreatitis	Abdominal fluid	*G. adiacens*
5	0140000, *A. adiacens*	2.5/F	Leukemia; trisomy 21	Blood	*G. adiacens*
6	0350451, *A. defectiva*	14/M	Cystic fibrosis	Blood	*A. defectiva*
7	0140020, *A. adiacens*	6/F	B-thalassemia major	Blood	*G. adiacens*
8	0350011, *A. defectiva*	7/F	Sickle cell disease	Blood	*A. defectiva*
9	0540000, *A. adiacens*	3/M	Leukemia	Blood	*G. elegans*
10	0350411, *A. defectiva*	6/M	Ependymoma	Blood	*A. defectiva*
11	0540000, *A. adiacens*	2/M	Hydrocephalus, ventriculoperitoneal shunt	CSF	*A. defectiva*
12	0140100, *A. adiacens*	0.5/F	Febrile infant; likely contaminant	Blood	*G. adiacens*
13	0350011, *A. defectiva*	9/F	Secondary leukemia after stem cell transplant	Blood	*G. adiacens*
14	Nonviable	6/M	Neuroblastoma	Blood	*G. adiacens*
15	0140100, *A. adiacens*	12/M	Renal failure	Blood	*G. elegans*

[a] Abbreviations: ID, identification; F, female; M, male; EVD, external ventricular drainage.

TABLE 3. Resistance of the clinical isolates to antimicrobial agents

Organism	No. of resistant isolates/total no. of isolates										
	Penicillin	Cefuroxime	Ceftriaxone	Erythromycin	Clindamycin	Tetracycline	Chloramphenicol	TMP-SMX[a]	Rifampin[b]	Ciprofloxacin[b]	Vancomycin
G. adiacens	2/6	6/6	6/6	4/6	2/6	2/6	0/6	0/6	0/6	0/6	0/6
G. elegans	0/3	2/3	2/3	1/3	1/3	0/3	0/3	0/3	0/3	1/3	0/3
A. defectiva	1/6	5/6	1/6	3/6	0/6	0/6	0/6	0/6	0/6	0/6	0/6
Total	3/15	13/15	9/15	8/15	3/15	2/15	0/15	0/15	0/15	1/15	0/15

[a] TMP-SMX, trimethoprim-sulfamethoxazole.
[b] See text for definition of resistance.

macrolides and clindamycin (12). For these organisms to date, there is only one fluoroquinolone-resistant isolate reported, from the blood of an adult febrile neutropenic patient who had received levofloxacin prophylaxis (13). All other isolates reported in three studies (12, 13, 19) were susceptible to levofloxacin. Among the 15 isolates in our study, the MICs of ciprofloxacin were low for 14, ranging from 0.25 to 1 μg/ml. One isolate for which the ciprofloxacin MIC was greater than 2 μg/ml was tested for levofloxacin by Etest, and the levofloxacin MIC was >32 μg/ml.

Macrolide resistance in streptococci can be caused by several different mechanisms. Ribosomal methylation (encoded by *erm* genes) and macrolide efflux [encoded by *mef*(A)] are among the most common mechanisms (11). Genotypic analysis by PCR for macrolide resistance genes was performed on all the isolates in this study (Table 2). Erythromycin-susceptible isolates were negative for these determinants tested. Among the eight resistant isolates, five were positive for *mef*(A) only and as expected were clindamycin susceptible. These included three *G. adiacens* and two *A. defectiva* isolates. Three other erythromycin-resistant isolates (two identified as *G. adiacens* and one as *G. elegans*) were positive for *erm*(B) and were also resistant to clindamycin, consistent with a constitutively expressed *erm*(B) gene. Interestingly, the *erm*(B)-positive *G. elegans* isolate was also positive for *mef*(A). The erythromycin MICs for these three *erm*(B)-positive isolates were 8 and >64 μg/ml.

The macrolide resistance observed in this small number of nutritionally variant isolates reflects the mechanisms and prevalence observed in *S. pneumoniae* and *Streptococcus pyogene*s in the United States. Efflux is the most common resistance mechanism among *S. pneumoniae* and *S. pyogene*s in the United States (60 to 70%), while *erm*(B) is less common (25%) (18, 18a). All three *erm*(B)-positive isolates were also *tet*(M) positive; two of the three were tetracycline resistant. For the third isolate (*G. elegans*) the tetracycline MIC was <1 μg/ml. The presence of *tet*(M) on this isolate was confirmed by DNA sequencing. In *S. pneumoniae erm*(B) and *tet*(M) are frequently found on the same transposon. Poyart et al. recently described similar findings in a clinical isolate of *A. defectiva* from a child with endocarditis (15). A Tn*916*-related element similar to the pneumococcal element Tn*3872* was found to be responsible for its erythromycin and tetracycline resistance. In the present study, we speculate that a similar mechanism here is highly possible although the presence and type of a transposon (if any) were not characterized. In addition, unlike the *A. defectiva* isolate in the previous report, the isolates in this study were identified as two of *G. adiacens* (both were negative for trehalose fermentation) and one of *G. elegans*, expanding our understanding of the resistance mechanisms in this group of bacteria.

Even though this represents a limited number of pediatric clinical isolates, our data suggest that the prevalence of beta-lactam and macrolide resistance is high among recent invasive isolates of nutritionally variant streptococci. In addition, we demonstrated for the first time that the macrolide efflux resistance mechanism encoded by *mef*(A) that is common in other streptococcal species is also found in these species and that the prevalence of *erm*(B) and/or *mef*(A) in nutritionally variant

TABLE 4. Macrolide and clindamycin susceptibilities and their genotypic determinants

Isolate no.	Identification	MIC (μg/ml)		Presence of determinant:			
		Erythromycin	Clindamycin	*erm*(A)	*erm*(B)	*mef*(A)	*tet*(M)
1	*G. elegans*	>64	>64	−	+	+	+
2	*G. adiacens*	>64	>64	−	+	−	+
3	*G. adiacens*	8[a]	≥256[a]	−	+	−	+
4	*G. adiacens*	8	0.25	−	−	+	ND[b]
5	*G. adiacens*	16	0.019	−	−	+	ND
6	*G. adiacens*	16	0.125	−	−	+	ND
7	*A. defectiva*	16	0.125	−	−	+	ND
8	*A. defectiva*	2	0.06	−	−	+	ND
Susceptible isolates (9–15)	*G. adiacens* (n = 2)	0.125 and <0.03	0.25 and 0.125	−	−	−	ND
	A. defectiva (n = 3)	≤0.125	≤0.125	−	−	−	ND
	G. elegans (n = 2)	≤0.094[a]	≤0.064[a]	−	−	−	ND

[a] Etest was used when poor growth in broth precluded assessment by microbroth dilution method.
[b] ND, not done.

TABLE 5. Comparison of resistance to some antimicrobial agents in different studies

Antimicrobial agent	Break point concn used (μg/ml)	% of isolates resistant			
		Present study (n = 15)	Tuohy et al. (19) (n = 39)	Murray et al. (13) (n = 20)	Liao et al. (12) (n = 28)
Penicillin	≥4	20	8	5	0
Ceftriaxone	≥4	60	13	35[a]	23[a]
Erythromycin	≥1	53		30	—[b]
Clindamycin	≥1	20	0	5	50
Tetracycline	≥8	13		10	
Chloramphenicol	≥16	0		0	
Vancomycin	>1	0	0	0	0

[a] Cefotaxime was described in the report.
[b] Azithromycin was tested in that report. For 57% of isolates, the MIC was ≥128 μg/ml, and only 7% of the isolates were susceptible.

streptococci parallels the reported distribution of these determinants in *S. pneumoniae* and *S. pyogenes*.

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