Mechanisms of Resistance for *Streptococcus pyogenes* in Northern Utah

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OBJECTIVE: The purpose of this study was to 1) determine the rates of penicillin and erythromycin resistance among *Streptococcus pyogenes* isolates in northern Utah, and 2) determine the genotype of the erythromycin resistant strains, thereby providing information regarding the mechanism of the resistance.

DESIGN: Seven hundred thirty-nine isolates of *S. pyogenes* were identified on 5% Sheep Blood Agar. Susceptibility to erythromycin and penicillin was performed using Muller-Hinton blood agar. All isolates resistant to erythromycin were then genotyped using PCR primers specific to one of the following: *mefA* gene, indicating the mechanism of resistance was an efflux pump; *ermA* gene, in which the mechanism was inducible methylation of the ribosomes; and *ermB* indicating constitutive methylation of the ribosomes.

LOCATION: This study was conducted at Weber State University, in the Department of Clinical Laboratory Sciences.

PATIENT SAMPLES: Samples were collected from 9 clinics ranging from North Ogden to Taylorsville, Utah. All samples were previously tested positive for *S. pyogenes* by the clinic from where the samples were collected.

RESULTS: Of the 739 *S. pyogenes* isolates tested, 2.4% were resistant to erythromycin with no resistance observed to penicillin. Of the strains that displayed some degree of resistance, the gene frequencies observed were as follows: 48.1% *mefA*, 26.0% *ermA*, 3.7% *ermB*, and 22.2% multiple genes.

CONCLUSION: The most common genotype was *mefA*, indicating that the efflux pump (M phenotype) is the most common mechanism in the surveyed area, followed by *ermA*, which produces the inducible methylating enzyme. A significant number of isolates was also observed to express both the efflux pump and the constitutive methylating enzyme.

ABBREVIATIONS: ATCC = American Type Culture Collection; CLS = clinical laboratory science; CLSI = Clinical Laboratory Standards Institute; IU = international unit; MLS_B = macrolide, lincosamide, streptogrammin B; PCR = polymerase chain reaction; SBA= sheep blood agar; WSU = Weber State University.

INDEX TERMS: erythromycin; erythromycin resistance mechanisms; PCR; penicillin; *S. pyogenes*.

Clin Lab Sci 2009;22(1):39

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ACKNOWLEDGEMENTS: *We would like to express our gratitude to the Clinical Laboratory Sciences Department faculty and staff for their endless help and support. We would also like to thank the clinics involved in the collection of samples for testing. We would like to thank our colleagues Todd Keskey, Bruce Jolly, Lauren Jackson, and Zach Robinette for helping us collect and isolate resistant strains. This project was funded through the Office of Undergraduate Research at WSU with a grant from the Denkers Undergraduate Research Scholarship.*

This information was presented as an oral presentation at the Weber State University Undergraduate Research Symposium and

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the ASCLS Utah Spring Seminar, April 2008, and as a poster presentation at the National Conference for Undergraduate Research in Salisbury MD in April 2008.

Streptococcus pyogenes is the most common cause of acute pharyngitis and can also be the cause of many other diseases, including bacteremia and necrotizing fasciitis. Because most strains are sensitive to penicillin, *S. pyogenes* infections are preferably treated with a drug from the penicillin family. If the patient is allergic to the drug, antimicrobials called macrolides, such as erythromycin, are used. Macrolide resistant strains of *S. pyogenes* have become a significant concern for healthcare providers. In Europe, studies have reported resistance to macrolides in more than 10% of strains tested from patients with *S. pyogenes* infections.[1] Studies conducted in Spain and Italy have reported resistance rates of 25% and 38%, respectively.[2,3] Over-prescription of macrolides is one suspected cause of this resistance.[4] Resistance in the United States has been reported as being somewhat lower than in European countries, ranging from three percent to nine percent.[5] Despite this relatively low rate, it continues to be a concern in the US due to the rapid increase of the resistance seen in Europe over just a few decades.

S. pyogenes resists the activity of macrolides via two predominant mechanisms. Some resistant strains utilize an efflux mechanism, where the bacterium utilizes a membrane associated protein that pumps the macrolide out of the cell, severely reducing the antimicrobial effect. This membrane protein is specific for macrolides. When the efflux mechanism is present in a macrolide resistant strain it is referred to as the M phenotype. *S. pyogenes* strains expressing the efflux pump are resistant to erythromycin, but they are susceptible to clindamycin (a drug from the lincosamide family) and streptogrammin B. Macrolides, lincosamides, and streptogrammin B are destructive to bacteria by binding to ribosomes and interfering with protein translation. The efflux pump (M phenotype) is associated with the presence of the *mefA* gene.[5]

The second mechanism of resistance is the modification of the bacterial ribosomes. Bacteria with this mechanism produce an enzyme that adds a methyl ($-CH_3$) group on the ribosome. This methylation slightly changes the shape of the ribosome and reduces the affinity of the drug for the ribosome. The characteristics displayed by this mechanism are referred to by the MLS_B phenotype.[6]

There are two types of MLS_B, inducible and constitutive. These MLS_B phenotypes can be easily distinguished by utilizing the D-test, or double disk diffusion test with a macrolide and a lincosamide. The inducible MLS_B requires the presence of a specific antimicrobial to induce the production of the enzyme. In the case of *S. pyogenes*, the macrolide erythromycin can elicit the production of the enzyme whereas the lincosamide clindamycin cannot. The bacterium is resistant to the lincosamide only in the presence of the enzyme inducing macrolide. Inducible MLS_B correlates with the *ermA* gene. The constitutive MLS_B mechanism continually produces the enzyme, and can therefore continually resist any ribosome targeting antimicrobial. Constitutive MLS_B expresses resistance to erythromycin and clindamycin, and is associated with the *ermB* gene.[5]

Typically, all of these mechanisms for macrolide resistance are represented in a given patient population. However, which mechanism is predominant varies from place to place. The efflux pump (*mefA* gene) is the most common in Spain.[2] A French pediatric study found 69.4% of the erythromycin resistance to be caused by the constitutive MLS_B *(ermB* gene)[7]. In the United States, the efflux pump (*mefA*) has been previously reported to account for 43%, while inducible MLS_B (*ermA*) accounts for 46%, with constitutive MLS_B (*ermB*) being much less at 8.5%.[1] The purpose of this study is to determine the rates of each mechanism of erythromycin resistance in northern Utah.

MATERIALS AND METHODS

Collection and isolation. Throat swabs were collected between October 2007 and March 2008 from 9 different clinics (Figure 1) covering a geographical area between North Ogden, and Taylorsville, Utah, which is about 50 miles south. The collection and isolation of *S. pyogenes* was performed in conjunction with another research group within the CLS department at WSU. All of the isolates were recovered from pharyngeal swabs collected from symptomatic patients. Clinic laboratories tested for *S. pyogenes* using point of care Strep tests. The swabs were then streaked onto five percent Sheep Blood Agar (SBA) plates (Hardy Diagnostics). Colonies that were translucent, β- hemolytic, and catalase negative were then subcultured to another SBA agar plate. Identification of the species was done using three criteria: β-hemolysis, susceptibility to bacitracin disk (0.04 IU), and a positive streptolysin-O stab.

Susceptibility testing. Susceptibility testing was performed using a Kirby Bauer disk diffusion method on Muller-Hinton agar plates, supplemented with five percent sheep blood (Hardy Diagnostics) according to CLSI M2-A9 guidelines.[8]

Each isolate was put into Columbia broth to a concentration of 0.5-0.9 McFarland units, and then swabbed onto Muller-Hinton blood agar bi-plates. A 10 IU penicillin and a 15 µg erythromycin disk were placed onto the swabbed area and then placed in an air incubator at 37°C for 16-24 hours.

After incubation, the zones of inhibition to each disk were measured using a micrometer. The size of the zone was used to determine if the strain was resistant (≤ 15 mm), intermediate (16-20 mm), or susceptible (≥ 21 mm) using the zones of inhibition listed by the antimicrobial manufacturer.

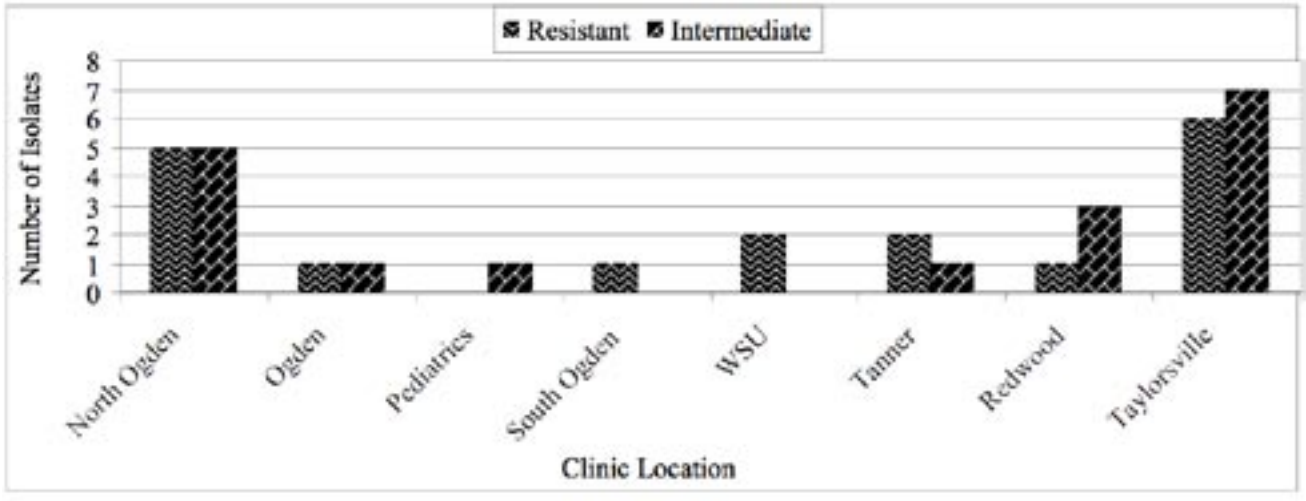
Controls. Several control strains were used in this study. For the bacitracin disks, *S. pyogenes* ATCC 19615 was used for the susceptible control, and *Streptococcus agalactiae* ATCC 12386 for the resistant control (MicroBiologics.com). For the erythromycin and penicillin susceptible control, *S. pyogenes* ATCC 19615 was used. For the erythromycin resistant controls representing each mechanism, strains of *S. pyogenes* were provided courtesy of the Doern research laboratory at the University of Iowa. There was no resistant control for penicillin.

Phenotyping. For the D-test, erythromycin resistant samples were once again put into a Columbia broth to a concentration of 0.5-0.9 McFarland units. The samples were swabbed onto a Muller-Hinton blood agar plate. The macrolide used for this test was a 15 µg erythromycin disk and the lincosamide used was a 2 µg clindamycin disk. The disks were placed 16-21 mm apart from each other, measuring from the center of each antimicrobial disk. Plates for the D-test were placed in the air incubator at 37°C for 16-24 hours. Again, the size of the zone of inhibition was used to determine if the strain was resistant, intermediate, or susceptible using the zones of inhibition included with the antimicrobials. If the isolate was resistant to erythromycin (≤15 mm) and susceptible to clindamycin (≥ 19mm), we recorded it as the M phenotype (efflux pump). The presence of a D shape around the susceptible region of clindamycin was the inducible MLS_B phenotype. Resistance to erythromycin (≤15 mm) and clindamycin (≤15 mm) was recorded as the constitutive MLS_B phenotype.

DNA extraction. DNA was extracted from strains showing a zone that was measured to be either intermediate or resistant to erythromycin. The extraction was performed using Qiagen QIAamp® DNA mini kit, following the manufacturer's protocol for DNA extraction from bacterial colonies.[9]

PCR amplification and detection. PCR amplification was used to detect *mefA, ermA*, and *ermB* genes. Previously published primers (*mefA,* 5'-AGT ATC ATT AAT CAC TAG TGC-3' and 5'-TTC TTC TGG TAC TAA AAG TGG-3'; *ermA,* 5'-GCA TGA CAT AAA CCT TCA-3' and 5'- AGG TTA TAA TGA AAC AGA-3'; and *ermB,* 5'-CGA GTG AAA AAG TAC TCA ACC-3' and 5'-GGC GTG TTT CAT TGC TTG ATG-3') [5] and thermal cycler conditions[1] were used. Each extracted sample was tested for each of the three genes separately. The PCR reaction concentrations consisted

Figure 1. Number of erythromycin resistant and intermediate strains listed by clinic location



of the following: 20 pM primers, 200 µM of each deoxynucleotide, 1.5 mM $MgCl_2$, 160 mM $(NH_4)_2SO_4$, 670 mM Tris-HCl (pH 8.8), 0.1% Tween-20, 2.5 U DNA polymerase (Bioline), and 1 µL of extracted DNA. Amplification products were separated by electrophoresis on a 1% agarose gel (150V for 30 min), and stained with ethidium bromide. The presence of the gene was determined by the presence of bands with the expected molecular sizes of 348, 206, and 616 base pairs for *mefA, ermA*, and *ermB* genes, respectively.[5] Control strains were used to confirm the accuracy of the PCR amplification: *S. pyogenes* 8084 for *mefA, S. pyogenes* 609 for *ermA*, and *S. pyogenes* 6945 for *ermB* (provided courtesy of the Doern research laboratory at the University of Iowa).

RESULTS

A total of 739 *S. pyogenes* isolates were identified and tested. Of these there were 36 that showed some degree of resistance. There were 18 resistant isolates, and 18 intermediate isolates (Figure 1). This equals a resistance rate of 2.4% and an intermediate rate of 2.4% (Table 1). All isolates showed susceptibility to penicillin.

Table 1. Number of *S. pyogenes* isolates collected from each clinic with number of erythromycin resistant and intermediate isolates

	Tested Samples				
	Isolates	**Resistant**	**Resistance (%)**	**Intermediate**	**Intermediate (%)**
North Ogden	98	5	5.1%	5	5.1%
Ogden	134	1	0.7%	1	0.7%
Pediatric	36	0	0.0%	1	2.8%
South Ogden	41	1	2.4%	0	0.0%
Weber State	6	2	33.3%	0	0.0%
Davis	7	0	0.0%	0	0.0%
Tanner	142	2	1.4%	1	0.7%
Redwood	108	1	0.9%	3	2.8%
Taylorsville	166	6	3.6%	7	4.2%
Total	739	18	2.4%	18	2.4%

Genotypic observations. After amplification, 27 isolates out of 36 showed the presence of at least one of the three probed genes. Of those 27, 13 displayed only the *mefA* gene (48.1%), 7 displayed only the *ermA* gene (26.0%), 1 displayed only the *ermB* gene (3.7%), 4 displayed both the *mefA* and *ermB* genes (14.8%), 1 displayed both the *mefA* and *ermA* genes (3.7%), and 1 displayed all three genes (3.7%). One resistant sample that showed constitutive MLS_B phenotypic characteristics had no gene amplified (see Figure 2 and Figure 3). There were eight intermediate isolates that did not show the presence of any gene after PCR amplification.

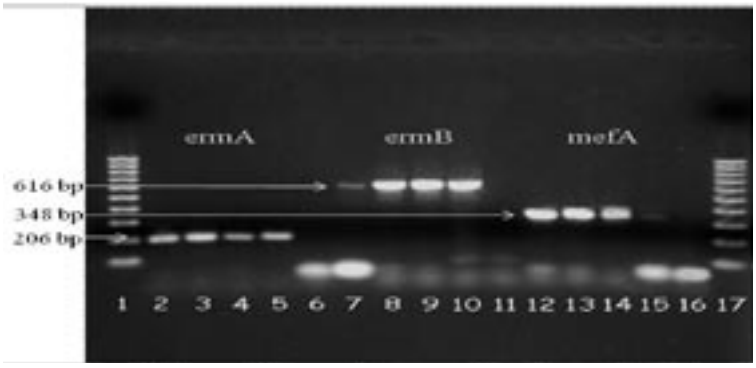
Phenotypic observations. Phenotypes were measured and recorded on 11 of the 36 erythromycin resistant and intermediate isolates. There were four samples (36%) that expressed the M phenotype (erythromycin resistant and clindamycin susceptible). There were four samples (36%) that displayed the inducible MLS_B phenotype (erythromycin resistant and inducible clindamycin resistance). The remaining three samples (27%) displayed the constitutive MLS_B phenotype (resistant to both erythromycin and clindamycin). The positive correlation between the phenotypes and genotypes of these samples are as follows: M phenotype, three of four contained the *mefA* gene (75%); inducible MLS_B phenotype, three of four contained the *ermA* gene (75%); constitutive MLS_B phenotype, one of three contained the *ermB* gene (33%). Of the 11 isolates with recorded phenotypes, 2 erythromycin

intermediate isolates failed to amplify any gene. Their phenotypes were documented as M and inducible MLS_B, respectively. There were two samples that amplified a gene other than the one predicted by the phenotype.

DISCUSSION

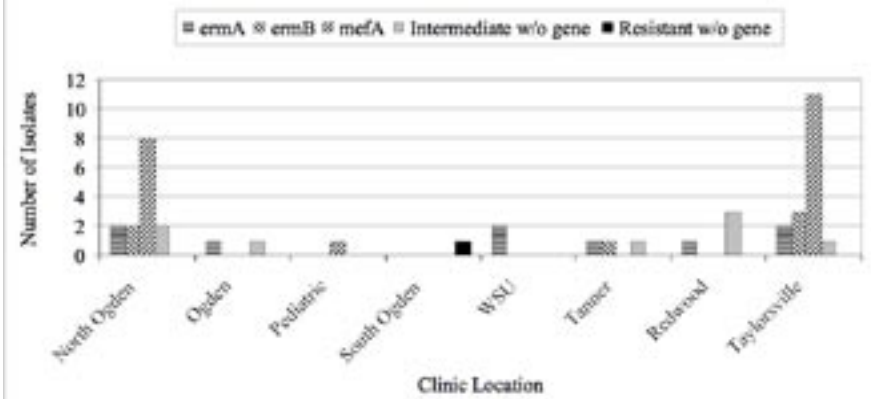
A significant finding from this study was that all *S. pyogenes* isolates were susceptible to penicillin, verifying its continued effectiveness against the organism. Previous studies have also reported little to no penicillin resistance in *S. pyogenes.*[5] The results show that the efflux pump (*mefA* gene) was the most prevalent mechanism (48.1%) of the erythromycin resistant strains. This is higher than the percent found in a previous published study done nationwide. However, the percent of the inducible MLS_B (*ermA*) was found to be lower in northern Utah than nationwide.[5]

Figure 2. Composite DNA electrophoresis gel



Composite gel photographed with all genes present. Lanes 1 and 17 are molecular weight markers for size determination of amplification products. Lanes 2, 7, and 12 are positive controls for each represented gene. Lanes 6, 11, and 16 are negative DNA PCR controls.

Figure 3. Genotype frequency of erythromycin resistant and intermediate strains by geographical location

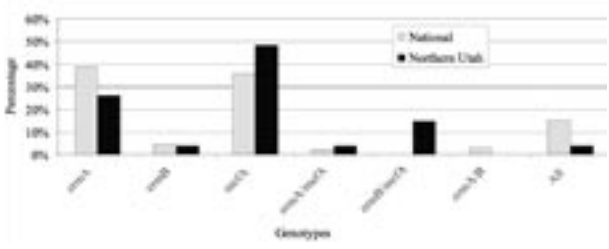


Some samples also showed multiple genes. Pairing of *ermA/mefA* and *ermB/mefA* in this study was observed at a higher percent than the national average (Figure 4). This survey also saw a lower percent of *ermA/ermB* pairings than the national average, as well as a decrease in the rate of isolates showing all three genes compared to the national average.

The national study showed only 7 samples out of 256 collected from the mountain area to be resistant to macrolides, displaying a 2.7% resistance rate. The same national survey showed that from 129 resistant strains collected nationwide, 11 showed the presence of *ermB* for an 8.5% occurrence whether paired with another gene or not.[5]

In this current study, an increased percentage of resistant strains was observed presenting the *ermB* gene (31.6%), contrasting the rate (8.5%) found in the national study (Figure 4). This may be noteworthy due to the fact that *ermB* correlates with the constitutive MLS_B phenotype, which is the only known erythromycin resistant mechanism that expresses resistance to lincosamides and streptogrammin B without the inducing macrolide present. An increased frequency of this gene could limit the antimicrobials that healthcare providers use in the case of invasive streptococcal infection. The overall erythromycin resistance rate of 2.4% is low enough to suggest that erythromycin and other macrolides are still viable options in the treatment of *S. pyogenes* for patients with penicillin allergies. Efflux (*mefA*) and inducible MLS_B (*ermA*) are the primary mechanisms causing the resistance in northern Utah. Constitutive MLS_B (*ermB*) was present in 18.5% of resistant isolates, making it a significant contributor to the resistant population as well.

Figure 4. Genotype frequency comparison



Because each mechanism is well represented in the resistant population, potential for expansion exists for each type. Continued utilization of penicillin as the first choice in the treatment of *S. pyogenes* will likely help to limit the potential for increased macrolide resistance in northern Utah.

There were eight intermediate isolates that did not show the presence of any gene after PCR amplification, and one strain that showed constitutive MLS_B phenotype, also with no gene product detected. One possible explanation for this may be due to another, less frequently encountered mechanism that was not included in this study.[5]

CONCLUSION

The rates of resistance for *S. pyogenes* to erythromycin in the northern Utah area are still at a relatively low level. Penicillin based antimicrobials should still be the first drug of choice when prescribing antimicrobials for treatment against this organism. The most common mechanism responsible for the resistance in the population of patients surveyed in this study was the efflux pump due to the presence of *mefA* gene (M phenotype). Coupling of *mefA* and *ermB* genes (efflux with constitutive ribosomal modification) suggests a greater presence of constitutive MLS_B phenotype will be seen in clinical settings when testing for mechanisms using the D-test. Knowing the mechanisms of resistance is useful in determining how to counteract the resistance and will influence the development and future administration of antimicrobials.

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