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In Vitro Development of Resistance to Telithromycin (HMR 3647), Four Macrolides, Clindamycin, and Pristinamycin in *Streptococcus pneumoniae*

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The ability of 50 sequential subcultures in subinhibitory concentrations of telithromycin (HMR 3647), azithromycin, clarithromycin, erythromycin A, roxithromycin, clindamycin, and pristinamycin to select for resistance was studied in five macrolide-susceptible and six macrolide-resistant pneumococci containing *mefE* or *ermB*. Telithromycin selected for resistance less often than the other drugs.

Ketolides are semisynthetic derivatives of erythromycin A characterized by a lack of α-L-cladinose on position 3 of erythronolide A, having a three-keto function. Telithromycin (HMR 3647) is a ketolide that has been shown to have good in vitro activity against penicillin- and erythromycin-resistant pneumococci (1, 3, 7, 8).

In a previous study (6) designed to determine if the recent dramatic increase in incidence of drug-resistant pneumococci may be due in part to abuse of oral drugs, such as macrolides and cephalosporins, we found that sequential subcultures in subinhibitory concentrations of azithromycin (used to represent the macrolide group), cefuroxime, and cefaclor lead to increased pneumococcal MICs. Since repeated exposure to azithromycin readily selected for pneumococci with increased azithromycin MICs, we investigated how pneumococcal MICs were affected by repeated exposure to a ketolide (an alternate choice to macrolides in empiric therapy of pneumococcal infections), in comparison to other antibiotics of the macrolide, lincosamide, and streptogramin class (MLS). Specifically, we repeatedly exposed 11 strains of *Streptococcus pneumoniae* to subinhibitory concentrations of telithromycin, azithromycin, clarithromycin, erythromycin A, roxithromycin, clindamycin, and pristinamycin to determine if resistance developed.

Five strains were susceptible to erythromycin A (MICs ≤ 0.25 μg/ml), and six were erythromycin A resistant: three strains had *mefE* (erythromycin A MICs, 2 to 4 μg/ml) and three strains had *ermB* (erythromycin A MICs, >64 μg/ml). Antimicrobials were obtained from their respective manufacturers.

MICs were determined by a standardized microdilution methodology in Mueller-Hinton broth (Difco Laboratories) supplemented with 5% lysed horse blood (4). Susceptibility breakpoints were those approved by the National Committee for Clinical Laboratory Standards (5): azithromycin, ≤0.5 μg/ml; clarithromycin, ≤0.25 μg/ml; erythromycin A, ≤0.25 μg/ml; and clindamycin, ≤0.25 μg/ml. The proposed susceptible breakpoint for telithromycin is ≤1 μg/ml (A. L. Barry, P. C. Fuchs, and S. D. Brown, Programs Abstr. Fourth Int. Conf. Macrolides, Azalides, Streptogramins Ketolides, abstr. 1.01, 1998). There are no roxithromycin and pristinamycin National Committee for Clinical Laboratory Standards breakpoints for *S. pneumoniae*. Daily passaging in subinhibitory concentrations of antibiotic for a maximum of 50 days was performed as published previously (6).

Resistant mutants were derived from parent strains as evidenced by (i) serotyping using the standard Quellung method with sera from Statens Seruminstitut (Copenhagen, Denmark) and (ii) pulsed-field gel electrophoresis using a CHEF DR III apparatus (Bio-Rad, Hercules, Calif.) as published previously (6). Strains were checked for the presence of *ermB* and *mefE* by amplification by PCR using primers and cycling conditions as described by Sutcliffe et al. (9).

MIC results from subculturing in subinhibitory concentrations of antibiotics are summarized in Table 1. Among the five macrolide-susceptible pneumococci, subculturing in telithromycin selected for two mutants, subculturing in pristinamycin selected for three mutants, subculturing in azithromycin, clarithromycin, and clindamycin selected for four mutants, and subculturing in erythromycin A and roxithromycin selected for mutants in all five strains. Among the three pneumococci containing *mefE*, subculturing in all the drugs led to selection of mutants with increased MICs in all three strains. The three pneumococci containing *ermB* were highly resistant to all the macrolides and clindamycin and, therefore, were subcultured only in telithromycin and pristinamycin. Among these three strains, subculturing in telithromycin and pristinamycin led to selection of mutants with increased MICs in all three strains.

Resistance was stable (MIC of passaged strains remained within one doubling dilution of MIC after 10 passages on antibiotic-free media), in most cases, among mutants derived from macrolide-susceptible parent strains and *ermB*-containing parent strains. In contrast, resistance was not stable among mutants derived from parent strains containing *mefE*. In these mutants, telithromycin and macrolide MICs usually reverted back to baseline MICs (or close to) after 10 passages on antibiotic-free media.

There were 54 mutants with elevated MICs to at least one of the antibiotics. Of these mutants, 3 were resistant to telithromycin (MICs >1 μg/ml), 20 were resistant to clindamycin (MICs ≥0.5 μg/ml), 36 were resistant to azithromycin (MICs ≥1 μg/ml), 37 were resistant to clarithromycin (MICs ≥0.5 μg/ml), 39 were resistant to erythromycin A (MICs ≥0.5 μg/ml), 45 had roxithromycin MICs of ≥0.5 μg/ml, and 20 had pristinamycin MICs of ≥1.0 μg/ml (Table 2). Macrolide-resistant mutants usually were still susceptible to telithromycin

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TABLE 1. Resistance selection results

Strain	Drug[a]	Initial MIC (μg/ml)	Selected resistance[b]		Retest MIC after 10 antibiotic-free subcultures[c]							Resistance genes[d]
			MIC	No. of subcultures	Az	Clar	Ery	Rox	Tel	Pris	Clin	
1	Az	0.03	>0.5	33	0.5	0.125	0.25	0.5	0.06	1	0.06	None
	Clar	0.015	>0.5	5	>32	16	>32	>32	1	0.5	1	None
	Ery	0.03	>0.5	12	2	0.5	0.5	1	0.008	0.5	0.125	None
	Rox	0.06	>1	9	0.06	0.03	0.06	0.125	0.008	0.5	0.03	None
	Tel	0.008	0.5	44	0.25	0.25	0.5	2	0.25	2	0.03	None
	Pris	0.5	NR	—	—	—	—	—	—	—	—	None
	Clin	0.03	1	28	0.5	0.03	0.06	0.125	0.008	0.5	0.5	None
2	Az	0.06	>0.5	28	0.5	0.06	0.125	0.125	0.008	0.5	2	None
	Clar	0.03	>0.5	15	>32	32	>32	>32	4	1	1	None
	Ery	0.06	0.5	24	0.125	0.06	0.125	0.25	0.03	0.5	0.25	None
	Rox	0.125	>1	23	0.25	0.25	0.5	1	0.06	0.5	0.125	None
	Tel	0.008	>0.5	24	>32	>32	>32	>32	8	2	0.125	None
	Pris	0.25	2	12	0.125	0.06	0.125	0.125	0.008	4	0.125	None
	Clin	0.125	0.5	16	0.06	0.015	0.06	0.125	0.008	0.25	0.125	None
3	Az	0.03	NR	—	—	—	—	—	—	—	—	None
	Clar	0.015	>0.5	13	>32	>32	>32	>32	0.25	0.5	2	None
	Ery	0.03	>0.5	16	4	16	>32	>32	0.25	2	0.125	None
	Rox	0.06	1	22	0.5	0.5	1	2	0.125	1	0.06	None
	Tel	0.008	NR	—	—	—	—	—	—	—	—	None
	Pris	0.5	2	22	0.03	0.015	0.03	0.06	0.004	0.5	0.125	None
	Clin	0.03	0.5	25	0.125	0.03	0.06	0.125	0.008	0.5	0.5	None
4	Az	0.015	>0.5	26	0.5	0.06	0.125	0.06	0.015	0.5	2	None
	Clar	0.015	>0.5	25	4	8	32	>32	0.125	4	0.25	None
	Ery	0.015	0.5	35	0.125	0.125	0.25	0.5	0.06	1	0.125	None
	Rox	0.03	>1	12	>32	2	8	16	0.015	0.25	2	None
	Tel	0.004	NR	—	—	—	—	—	—	—	—	None
	Pris	0.25	2	28	0.06	0.125	0.125	0.5	0.125	2	0.03	None
	Clin	0.03	>1	27	0.5	0.06	0.06	0.5	0.015	0.5	2	None
5	Az	0.015	>0.5	24	>32	>32	>32	>32	0.06	0.5	4	None
	Clar	0.015	NR	—	—	—	—	—	—	—	—	None
	Ery	0.015	0.5	48	0.125	0.25	0.25	1	0.125	2	0.03	None
	Rox	0.06	1	35	0.06	0.125	0.25	0.5	0.125	2	0.03	None
	Tel	0.004	NR	—	—	—	—	—	—	—	—	None
	Pris	0.5	NR	—	—	—	—	—	—	—	—	None
	Clin	0.03	NR	—	—	—	—	—	—	—	—	None
6	Az	2	16	13	2	2	4	8	0.125	0.5	0.06	*mefE*
	Clar	2	16	10	4	2	4	16	0.06	0.5	0.03	*mefE*
	Ery	4	32	10	>32	16	32	>32	0.125	0.5	0.5	*mefE*
	Rox	8	>32	7	8	8	16	32	0.25	1	0.03	*mefE*
	Tel	0.125	1	7	2	2	4	8	0.125	0.5	0.03	*mefE*
	Pris	0.5	4	22	2	2	4	8	0.06	1	0.125	*mefE*
	Clin	0.03	0.5	12	2	2	4	8	0.125	0.5	0.5	*mefE*
7	Az	1	8	6	4	2	4	8	0.125	0.25	0.03	*mefE*
	Clar	1	>8	7	4	2	2	8	0.06	0.25	0.03	*mefE*
	Ery	2	16	7	2	1	2	4	0.06	0.25	0.03	*mefE*
	Rox	4	32	9	2	1	2	8	0.06	0.25	0.06	*mefE*
	Tel	0.06	>1	7	2	2	4	8	0.25	0.25	0.03	*mefE*
	Pris	0.25	2	21	4	2	4	16	0.125	1	0.03	*mefE*
	Clin	0.015	>1	19	1	1	2	8	0.06	0.5	2	*mefE*
8	Az	1	>8	6	1	1	2	4	0.06	0.25	0.03	*mefE*
	Clar	1	16	13	4	2	4	8	0.125	0.25	0.06	*mefE*
	Ery	2	16	6	2	1	2	4	0.125	0.25	0.03	*mefE*
	Rox	4	32	8	16	2	8	32	0.06	0.25	0.06	*mefE*
	Tel	0.06	1	7	2	1	2	8	0.06	0.25	0.03	*mefE*
	Pris	0.25	2	21	4	2	4	16	0.125	2	0.06	*mefE*
	Clin	0.03	0.5	19	2	1	2	4	0.06	0.5	0.5	*mefE*
9	Az	>64										
	Clar	>64										

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TABLE 1—*Continued*

Strain	Drug[a]	Initial MIC (μg/ml)	Selected resistance[b]		Retest MIC after 10 antibiotic-free subcultures[c]							Resistance genes[d]
			MIC	No. of subcultures	Az	Clar	Ery	Rox	Tel	Pris	Clin	
	Ery	>64										
	Rox	>64										
	Tel	0.125	>1	3	—	—	—	—	4	0.5	—	*ermB*
	Pris	0.5	4	8	—	—	—	—	0.125	1	—	*ermB*
	Clin	>16										
10	Az	>64										
	Clar	>64										
	Ery	>64										
	Rox	>64										
	Tel	0.03	1	6	—	—	—	—	1	2	—	*ermB*
	Pris	0.25	2	11	—	—	—	—	0.03	1	—	*ermB*
	Clin	>16										
11	Az	>64										
	Clar	>64										
	Ery	>64										
	Rox	>64										
	Tel	0.03	>1	6	—	—	—	—	0.03	0.5	1	*ermB*
	Pris	0.5	4	10	—	—	—	—	0.5	2	—	*ermB*
	Clin	>16										

[a] Az, azithromycin; Clar, clarithromycin; Ery, erythromycin A; Rox, roxithromycin; Tel, telithromycin; Pris, pristinamycin; Clin, clindamycin.
[b] NR, no increase in MIC detected.
[c] —, not determined.
[d] Strains checked for resistance genes *mefE* and *ermB*.

MICs (≤0.25 μg/ml). Mutants with telithromycin MICs of ≥1 μg/ml were cross resistant to all macrolides (MICs ≥16 μg/ml). In most cases, exposure of strains to clindamycin or pristinamycin selected mutants with increased MICs to the selecting drug while the MICs to telithromycin and the macrolides remained unchanged or slightly higher.

Pneumococcal mutants (derived from subculturing in azithromycin) from a previous study of ours (6) have recently been analyzed. Resistance was determined to be caused by (i) mutations in domain V of 23S rRNA leading to ML, MS, or MLS_B phenotypes, depending on the specific mutation and (ii) mutations in ribosomal protein L4 leading to an MS phenotype (A. Tait-Kamradt, T. Davies, M. Jacobs, P. Appelbaum, and J. Sutcliffe, Abstr. 39th Intersci. Conf. Antimicrob. Agents Chemother., abstr. C-0842, 1999). At least two of the four copies of the 23S rRNA genes needed to be mutated to see elevated macrolide MICs (Tait-Kamradt et al., 39th ICAAC, abstr. C-0842). Similar studies to determine the mechanisms of resistance among the mutants generated in this study are in progress.

The clinical significance of these in vitro studies recently became evident, as we have documented that macrolide resistance among some clinical pneumococcal strains can be caused by the same or similar ribosomal mutations as those observed in the in vitro studies. We found mutations in ribosomal protein L4 among several clinical strains of *S. pneumoniae* from Central and Eastern Europe (some clonally related) and mutations in domain V of 23S rRNA among a few recent U.S. clinical strains (A. Tait-Kamradt, T. Davies, L. Brennan, P. Depardieu, P. Courvalin, J. Duigan, J. Petitpas, L. Wondrack, M. Jacobs, P. Appelbaum, and J. Sutcliffe, Final Program, Abstr. Exhibits Addendum 39th Intersci. Conf. Antimicrob. Agents Chemother. abstr. LB-8, 1999). The relationship between this L4 clone and macrolide use in Central and Eastern Europe is unknown, but clearly these drugs are used on a large scale in many of these countries (W. Hyrniewicz, personal communication). Thus far, macrolide-resistant pneumococci from clinical specimens that do not have *mef* or *erm* genes are extremely rare (8; Hyrniewicz, personal communication). This study suggests that overuse and misuse of MLS agents could lead to an increase in the incidence of macrolide resistance among pneumococci (by either *mef* or *erm* genes or ribosomal

TABLE 2. Number of mutants (n = 54) with specific MICs to each of the antibiotics

Drug	MIC (μg/ml)[a]													
	0.004	0.008	0.015	0.03	0.06	0.125	0.25	0.5	1.0	2.0	4.0	8.0	16.0	≥32.0
Telithromycin	1	7	3	3	14	15	5	1	2	**—**	**2**	**1**	**—**	**—**
Azithromycin	—	—	—	1	4	5	2	6	**2**	**11**	**8**	**1**	**1**	**13**
Clarithromycin	—	—	2	3	5	4	3	**2**	**7**	**13**	**—**	**2**	**3**	**10**
Erythromycin A	—	—	—	1	5	5	4	**3**	**1**	**8**	**10**	**2**	**1**	**14**
Roxithromycin	—	—	—	0	2	6	1	**5**	**3**	**2**	**4**	**11**	**4**	**16**
Clindamycin	—	—	—	16	7	9	2	**5**	**2**	**6**	**1**	**—**	**—**	**6**
Pristinamycin	—	—	—	—	—	—	12	22	9	9	2	—	—	—

[a] —, no mutants with MIC. Numbers and dashes in bold represent resistance to the antibiotic.

mutations). The need for cautious and judicious use of antimicrobials for treatment of pneumococcal infections is warranted.

In summary, telithromycin has been shown to have good in vitro activity against strains containing *mefE* and *ermB* and against in vitro-selected mutants resistant to other macrolides, clindamycin, and pristinamycin. While exposure to telithromycin did select for pneumococcal mutants with increased MICs (most of which were still susceptible), it did so in the least number of strains, compared to the other MLS agents.

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