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Effect of Efflux on Telithromycin and Macrolide Susceptibility in *Haemophilus influenzae*

Tatiana Bogdanovich,[1] Bülent Bozdogan,[2] and Peter C. Appelbaum[1]*

Department of Pathology, Hershey Medical Center, Hershey, Pennsylvania 17033,[1] and Mikrobiyoloji ve Klinik Mikrobiyoloji AD, Adnan Menderes Universitesi Tıp Fakültesi, Aydın, Turkey 09100[2]

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This study investigated the presence of telithromycin and azithromycin efflux in 58 clinical strains of *Haemophilus influenzae* with various susceptibilities to macrolides, azalides, and ketolides. Efflux pumps were studied by measuring accumulation of radioactive [^{3}H]telithromycin and [*N*-methyl-^{3}H]azithromycin in the presence and absence of carbonyl *m*-chlorophenylhydrazone (CCCP), a protonophore. In 17 strains for which the telithromycin MICs were 0.06 to 0.5 μg/ml (azithromycin MICs, ≤0.06 to 0.125 μg/ml; clarithromycin MICs, ≤0.06 to 2 μg/ml), telithromycin and azithromycin accumulations were high without CCCP and not affected by its addition, which indicates absence of efflux. In 22 strains for which the telithromycin MICs were 0.25 to 4 μg/ml (azithromycin MICs, 0.25 to 1 μg/ml; clarithromycin MICs, 1 to 8 μg/ml), initially low levels of telithromycin accumulation became higher after addition of CCCP, indicating a functioning efflux pump. Nineteen strains for which the telithromycin MICs were ≥2 μg/ml had efflux as well as various mutations in ribosomal proteins L4, L22, and/or 23S rRNA (domains II and V). Of these 19 strains, the telithromycin MICs (≥8 μg/ml) for 17 of them were significantly raised (azithromycin, MICs 4 to >32 μg/ml; clarithromycin MICs, 8 to >32 μg/ml). From these results we conclude that telithromycin efflux with or without additional ribosomal alterations is present in all *H. influenzae* strains, except for those for which the telithromycin MICs were very low.

Haemophilus influenzae is one of the most common pathogens causing community-acquired respiratory tract infections, including community-acquired pneumonia and acute exacerbation of chronic bronchitis, which are associated with considerable morbidity, mortality, and high financial burden (1, 22, 17). *H. influenzae* is also frequently implicated in sinusitis and acute otitis media, which are usually less severe and non-life-threatening but have potential for serious complications if not treated properly (12, 7, 9). Macrolides, azalides, and ketolides are currently recommended for treatment of community-acquired pneumonia and acute exacerbations of chronic bronchitis, with quinolones such as levofloxacin and moxifloxacin being alternative agents (15, 16, 19).

H. influenzae demonstrates relatively good in vitro susceptibility to macrolides and azalides, which have a unimodal MIC distribution and low prevalence of high-level resistance when defined by current Clinical and Laboratory Standards Institute (CLSI) standards. Telithromycin appears to have less optimal and more variable activity against *H. influenzae* compared to azithromycin. Several in vitro studies have demonstrated that telithromycin MICs were equal to or at least 1 dilution higher than those of azithromycin (24). Telithromycin activity against *H. influenzae* is, however, superior to that of erythromycin and clarithromycin (14). MICs and breakpoints of macrolides, azalides, and ketolides against this organism must be considered together with their raised levels in tissue and epithelial lining fluids, which may improve their clinical efficacy in treatment of pneumonia (28). However, if pharmacological/pharmacodynamic breakpoints (24-h area under the curve/MIC > 25) are used, macrolides and ketolides should be ineffective against >95% of *H. influenzae* (10). This latter finding is supported by the high bacteriologic failure rates found in clinical trials of macrolides and ketolides for the treatment of acute exacerbations of chronic bronchitis and otitis media caused by *H. influenzae* (2, 3, 5, 6). It should also be noted that the exact pharmacokinetic/pharmacodynamic parameters for telithromycin have not yet been precisely defined (W. A. Craig, personal communication).

The main mechanisms of macrolide resistance include target modification (methylation of specific residues in 23S rRNA by methylases encoded by the *erm* class of genes or mutations in 23S rRNA and ribosomal proteins L4 and L22), active efflux of the drugs encoded by the *mef* gene, and, rarely, antibiotic inactivation (11).

Three susceptibility groups have been defined previously by our group among clinical *H. influenzae* strains with regard to their macrolide susceptibility (20): (i) macrolide hypersusceptible strains, accounting for less than 2% of *H. influenzae* strains, had no resistance mechanism for macrolides; (ii) baseline strains had only a macrolide efflux mechanism (both of the latter two groups were susceptible by CLSI criteria); (iii) in strains termed hyperresistant, alteration of ribosomal proteins L4 and L22 or 23S rRNA combined with intrinsic efflux mechanisms increased the level of MICs beyond the CLSI breakpoints for macrolides (18). Thus, relatively high telithromycin MICs for *H. influenzae* may indicate the presence of telithromycin efflux. The aim of this study was to test telithromycin susceptibility of *H. influenzae* isolates from different macrolide susceptibility groups and to verify the presence or absence of

* Corresponding author. Mailing address: Department of Pathology, Hershey Medical Center, P.O. Box 850, Hershey, PA 17033. Phone: (717) 531-5113. Fax: (717) 531-7953. E-mail: pappelbaum@psu.edu.

TABLE 1. MIC and radioactive accumulation results for *H. influenzae* strains[a]

Strain	TEL MIC	TEL efflux, accumulation[b]	AZI MIC	AZI efflux/accumulation[b]	CLA MIC
Telithromycin-susceptible strains with no efflux					
1999-211-038	0.06	(−)/0.3	0.125	(−)/−0.09	1
1999-211-085	0.06	(−)/0.48	0.125	(−)/0.21	1
1999-211-088	0.06	(−)/0.10	0.125	(−)/0.45	1
1999-211-098	0.06	(−)/0.04	0.125	(−)/0.16	1
SJ15	0.06	(−)/0.57	≤0.06	(−)/0.09	≤0.06
SJ6	0.06	(−)/−0.23	<0.06	(−)/−0.05	≤0.06
1998-117-108	0.125	(−)/0.30	0.125	(−)/0.74	1
1998-117-238	0.125	(−)/0.56	≤0.06	(−)/0.38	2
1999-130-002	0.125	(−)/−0.23	0.125	(−)/0.06	2
SJ5	0.125	(−)/0.40	≤0.06	(−)/0.08	0.25
SJ7	0.125	(−)/−0.11	≤0.06	(−)/−0.05	0.25
1999-110-0.26	0.25	(−)/0.23	0.125	(−)/0.54	2
2000-126-002	0.25	(−)/0.38	0.125	(−)/0.05	2
HH751	0.25	(−)/0.41	0.125	(−)/0.09	2
SJ19	0.25	(−)/0.49	0.125	(−)/0.28	0.5
SJ14	0.5	(−)/0.18	0.25	(−)/0.36	1
SJ16	0.5	(−)/0.37	0.25	(−)/0.16	1
Telithromycin-susceptible strains with efflux					
2001-130-005	0.25	(+)/2.29	0.25	(−)/0.43	1
HI30	0.25	(+)/1.94	0.125	(+)/1.06	0.5
HH477	0.5	(+)/1.23	0.25	(−)/0.74	1
HH722	0.5	(+)/2.59	0.125	(−)/0.88	2
HH746	0.5	(+)/1.29	0.125	(−)/0.11	2
MJ23	0.5	(+)/2.16	0.25	(−)/0.20	2
SJ1	0.5	(+)/1.93	0.25	(−)/−0.10	2
SJ2	0.5	(+)/1.20	0.25	(−)/0.06	4
2000-126-009	1	(+)/1.91	0.5	(+)/1.28	4
2000-126-018	1	(+)/4.01	0.5	(+)/1.41	4
2000-621-092	1	(+)/2.36	0.5	(−)/0.70	4
SJ12	1	(+)/2.14	0.25	(−)/0.67	2
SJ21	1	(+)/1.46	0.5	(−)/0.77	4
HH439	2	(+)/2.95	0.25	(+)/1.35	4
HH739	2	(+)/3.92	0.5	(+)/1.58	4
HH768	2	(+)/1.35	0.5	(−)/0.58	2
MJ12	2	(+)/1.81	0.5	(+)/1.14	4
MJ17	2	(+)/1.41	0.5	(−)/0.19	8
MJ22	2	(+)/1.93	1	(+)/4.8	8
1999-112-023	4	(+)/4.7	8	(+)/4.07	>32
MJ13	4	(+)/1.93	1	(+)/1.38	8
MJ3	4	(+)/1.79	8	(+)/0.89	>32
Telithromycin-resistant strains					
1999-130-053	8	(+)/2.67	8	(+)/1.55	>32
HH506	8	(+)/2.08	4	(+)/1.11	>32
MJ4	16	(+)/1.33	16	(+)/2.68	>32
MJ5	16	(+)/2.29	8	(+)/1.65	>32
MJ9	16	(+)/3.16	4	(+)/1.29	32
MJ16	32	(+)/1.28	16	(+)/1.75	>32
MJ19	32	(+)/4.10	8	(+)/5.83	32
MJ20	32	(+)/3.47	32	(+)/1.74	>32
MJ6	32	(+)/1.97	32	(+)/1.41	>32
1999-130-001	32	(+)/2.63	8	(+)/1.33	>32
HI100	64	(+)/2.29	>32	(+)/7.3	>32
MJ7	64	(+)/1.82	16	(+)/2.68	>32
1999-130-044	>64	(+)/5.01	>32	(−)/0.43	>32
MJ1	>64	(+)/3.29	>32	(+)/3.46	>32
MJ10	>64	(+)/1.55	>32	(+)/0.74	>32
MJ11	>64	(+)/3.99	>32	(+)/5.03	>32
MJ2	>64	(+)/1.99	32	(+)/2.49	>32
MJ24	>64	(+)/1.85	>32	(−)/0.71	>32
MJ8	>64	(+)/1.85	32	(+)/1.89	>32

[a] TEL, telitromycin; AZI, azithromycin; CLA, clarithromycin.

[b] Ratio between the radioactive counts with CCCP and those without CCCP after 30 min of exposure to radioactive antibiotic, and 1 was subtracted from the ratio to normalize the no-change value to 0 (20).

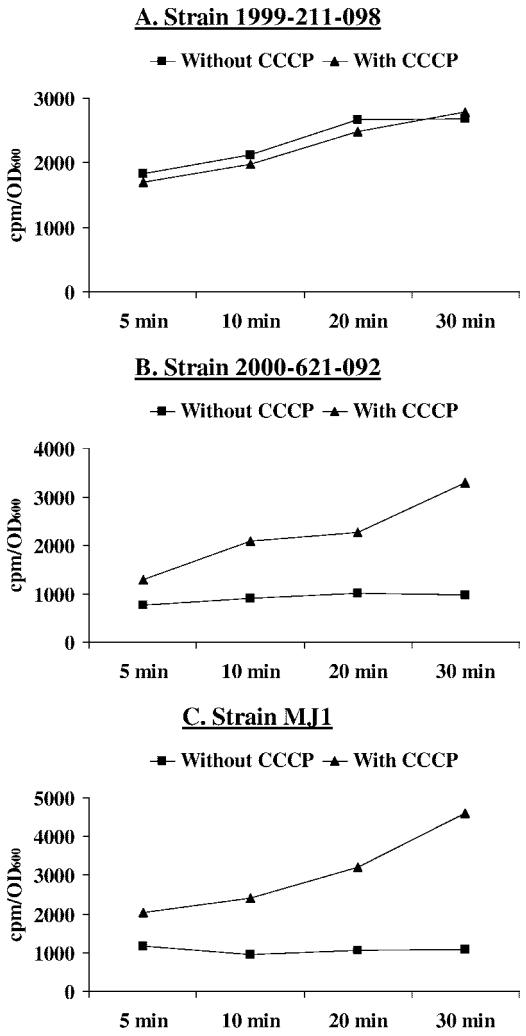

FIG. 1. Accumulation of radioactive telithromycin in a telithromycin-susceptible strain with no efflux (A), a telithromycin-susceptible strain with efflux (B), and a telithromycin-resistant strain (C). cpm, counts per minute.

an efflux pump in *H. influenzae*, which affects telithromycin accumulation and susceptibility.

MATERIALS AND METHODS

Susceptibility testing. Clinical *H. influenzae* strains from previous macrolide and azalide efflux studies (20), and additional strains collected within the Alexander Project (1997 to 2001) were included in the present work to represent groups with various telithromycin susceptibility patterns. MICs were tested by the Clinical and Laboratory Standards Institute (CLSI) macrodilution method using freshly prepared *Haemophilus* test medium (HTM) (18). Telithromycin, azithromycin, and clarithromycin powders were obtained from their respective manufacturers. Inoculum was prepared from cultures grown on chocolate agar plate for 16 to 18 h. The standard quality control strain of *H. influenzae* ATCC 49247 was used on each day of testing. A growth control tube with no antibiotic was also included to assess the ability of *H. influenzae* strains to grow in HTM. Macrodilution tubes were incubated at 35°C for 20 to 24 h in ambient air.

Efflux assays. Telithromycin and azithromycin efflux were determined indirectly by measuring the accumulation of radioactive [^{3}H]telithromycin (Moravek Biochemicals) and [*N*-methyl-^{3}H]-azithromycin (Perkin-Elmer Life Sciences) as described previously (20). Briefly, 40 ml of freshly made HTM was inoculated with 2 ml of an overnight culture of the selected strain and grown at 35°C. After the optical density at 600 nm (OD_{600}) reached 0.35 to 0.4, half of the culture was exposed to carbonyl cyanide *m*-chlorophenylhydrazone (CCCP; 25 μg/ml) for 10 min, and 2.3 μg of radioactive antimicrobial was then added to both samples. Four milliliters of culture was removed from each sample at 5, 10, 20, and 30 min and filtered through a Whatman GF/C glass microfiber filter previously wetted with saline containing 1 μg/ml of corresponding unlabeled antimicrobial. Filters were washed twice with saline-drug mixture and dried at room temperature. Radioactivity was then determined by liquid scintillation counting. Radioactive accumulation was expressed as the ratio between the radioactive counts with CCCP and those without CCCP after 30 min of exposure to radioactive antibiotic. In an effort towards consistency with our previous publication (20), 1 was subtracted from the ratio to normalize the no-change value to 0.

DNA amplification and sequencing. The presence of macrolide resistance genes [*erm*(A), *erm*(B), *mef*(A), and *ere*(A)] and mutations in the genes coding for ribosomal proteins L4 and L22 and domain V of 23S rRNA was studied as described previously (25, 20, 21). For domain II of 23S rRNA the following primers were used: Hin23S-547F (5′-TTC AGC CCC GTT ACA TCT TC-3′) and Hin23S-1144R (5′-TTC AGC CCC GTT ACA TCT TC-3′). Template DNA for PCR was prepared using InstaGen Matrix, as recommended by the manufacturer (Bio-Rad Laboratories, Hercules, CA). After amplification PCR products were purified from excess primers and nucleotides using a QIAquick PCR Purification kit (QIAGEN, Valencia, CA) and sequenced directly using CEQ8000 Genetic Analysis System (Beckman Coulter, Fullerton, CA).

RESULTS

Susceptibility of *H. influenzae* to macrolides and telithromycin. MIC testing results are shown in Table 1. Analysis of susceptibilities of 58 tested strains using current CLSI breakpoints (18) showed that 39 (67.2%) strains were telithromycin susceptible (MIC range, 0.06 to 4 μg/ml), 2 (3.5%) were intermediate (MIC, 8 μg/ml), and 17 (29.3%) were resistant (MIC, 16 to >64 μg/ml). For macrolides, 39 (67.2%) and 37 (63.8%) were susceptible to azithromycin (MIC range, <0.06 to 4 μg/ml) and clarithromycin (MIC, ≤0.06 to 8 μg/ml), respectively; 19 (32.8%) and 21 (36.2%) strains were not susceptible to azithromycin (MIC, >4 μg/ml) and clarithromycin (MIC, >8 μg/ml), respectively.

Telithromycin efflux assay. In 17 *H. influenzae* strains with telithromycin MICs ranging between 0.06 and 0.5 μg/ml, accumulation was high without CCCP and was not affected by its addition, which indicates the absence of telithromycin efflux (Table 1, Fig. 1A).

In 22 strains with telithromycin MICs of 0.25 to 4 μg/ml (susceptible by current CLSI breakpoints) and in 19 strains with significantly raised telithromycin MICs (≥8 mg/ml), initially low levels of telithromycin accumulation became higher after addition of CCCP, indicating the presence of efflux mechanisms (Fig. 1B and C). All of the latter strains as well as five strains for which the telithromycin MICs were 2 to 4 μg/ml also had various mutations in ribosomal proteins L4, L22, and/or 23S rRNA (Table 2).

Accumulation of radioactive telithromycin in all 58 tested strains is shown in Table 1. The mean of the ratio for telithromycin-susceptible isolates without efflux was 0.27; for those strains susceptible by CLSI but with efflux the mean value was 2.15, and for strains resistant by CLSI it was 2.52. These differences were statistically significant for strains susceptible with efflux versus those without efflux as well as for resistant versus susceptible strains without efflux ($P < 0.001$). There was no statistical difference between the telithromycin accumulation values for strains susceptible with efflux and resistant strains ($P = 0.69$).

Correlation of susceptibility and efflux to telithromycin and macrolides. When azithromycin and clarithromycin suscepti-

TABLE 2. MICs for and detected mutations in *H. influenzae* strains

Strain	MIC (μg/ml) of:			Mutation(s)			
	Telithromycin	Azithromycin	Clarithromycin	L4	L22	23S rRNA	
						Domain II	Domain V
MJ17	2	0.5	8	A69S		G845C, U855C, C894A, A925G	
MJ22	2	1	8	K61Q		G884U, C894A	
1999-112-023	4	8	>32	G65C		A654G	
MJ13	4	1	8	K61Q		A633G, A654G	
MJ3	4	8	>32	D139G		G884U, C894A	G2160U
1999-130-053	8	8	>32	G65D		A654G, INS G877	
HH506	8	4	>32			A654G	
MJ4	16	16	>32	K61Q		C612U, A654G	
MJ5	16	8	>32	T64K	G91D	A654G, INS A602, INS A624	C2164G
MJ9	16	4	32		DEL 81S	A654G, G884U, C894A	
MJ16	32	16	>32		INS 91 KG	C635G	
MJ19	32	8	32	K61Q		G884U, C894A	
MJ20	32	32	>32		DEL M82	G884U, C894A	
MJ6	32	32	>32	T64K		C612U, A654G	
1999-130-001	32	8	>32		DEL 96ILK	G884U, C894A	
HI100	64	64	>32	K61Q		A654G, G884U, C894A	
MJ7	64	16	>32		DEL 95RI	G884U, C894A	
1999-130-044	>64	>32	>32		INS 88RAKG	A654G	
MJ1	>64	>32	>32		DEL 95RI	C885U, C894A	GGA2160-2UAU
MJ10	>64	>32	>32			A654G	A2058G, G2160U
MJ11	>64	>32	>32	T64K	G91D	G884U, C894A	
MJ2	>64	32	>32	T64K	G91D	A654G	C2164G
MJ24	>64	>32	>32	T64K	G91D	A654G	
MJ8	>64	32	>32	T64K	G91D	A654G	C2164G

bility was analyzed with respect to telithromycin susceptibility and the presence of telithromycin efflux, the following results were obtained. For telithromycin-susceptible strains without telithromycin efflux, azithromycin and clarithromycin MICs ranged between ≤0.06 to 0.25 and ≤0.06 to 2 μg/ml, respectively. All telithromycin-susceptible strains lacking telithromycin efflux pumps were also negative in the azithromycin efflux assay (Table 1).

For telithromycin-susceptible strains with telithromycin efflux, azithromycin and clarithromycin MICs were 0.125 to 8 μg/ml and 0.5 to >32 μg/ml, respectively. Of these 22 strains, 10 isolates (azithromycin MIC range, ≤0.125 to 8 μg/ml) had an azithromycin efflux pump. The azithromycin MICs for the remaining 12 strains were 0.125 to 0.5 μg/ml, and the strains were negative in the azithromycin efflux assay (Table 1).

Azithromycin and clarithromycin MICs for telithromycin-resistant strains were 4 to >32 μg/ml and >32 μg/ml, respectively. With the exception of two strains where azithromycin efflux results were not convincing, all telithromycin-resistant *H. influenzae* strains had an azithromycin efflux pump present (Table 1).

DISCUSSION

Similar to our previous report (20), three distinct telithromycin susceptibility groups could be defined among clinical strains of *H. influenzae*: (i) telithromycin-susceptible strains (CLSI) with no evidence of telithromycin efflux; (ii) telithromycin-susceptible strains (CLSI) with efflux; (iii) telithromycin-resistant strains with efflux as well as various ribosomal alterations. Previous reports have shown that the macrolide-ketolide antibiotic binding site is formed by structures in domains II and V of 23S rRNA (8, 27) and that mutation U754A in hairpin 35 of domain II of 23S rRNA confers resistance to macrolides and ketolide (27). In contrast, in our study none of the telithromycin-resistant strains had the latter mutation while other mutations, including A654G, were found in the domain II of resistant strains. Involvement of domain II mutations is currently being studied.

Like many other gram-negative bacteria, *H. influenzae* is known to possess active efflux transporters. Previously, Sanchez et al. found that the *acrAB* homolog of *H. influenzae* codes for a functional multidrug system pump (23). This pump belongs to the resistance nodulation cell division family and utilizes an electrochemical potential of H^+ across cell membrane as its driving force. Inactivation of AcrAB increased susceptibility of *H. influenzae* to various antimicrobials including erythromycin, dyes, and detergent. Later, Trepod et al. studied substrate specificities of 10 putative efflux pump in *H. influenzae* (26) and showed that TolC protein acts together with AcrA and AcrB to form a single primary efflux pump for *H. influenzae*. Interestingly, in contrast to the *Escherichia coli* AcrAB system, the AcrAB/TolC pump of *H. influenzae* did not expel chloramphenicol, tetracycline, and fluoroquinolones (23, 26). Sanchez et al. speculated that rapid influx of the latter small antibiotic molecules through the large *H. influenzae* porin channels counterbalances their efflux (23). Data have been presented indicating that deletion of the AcrB gene resulted in a significant decrease in MICs to both azithromycin and telithromycin, indicating that this pump is responsible for the efflux of both azithromycin and telithromycin (J. Sutcliffe, Abstr. 43rd Intersci. Conf. Antimicrob. Agents Chemother., abstr. 1336, 2003). However, Peric et al. could not find significant differences in either sequence or expression level for

acrAB gene clusters in high-level macrolide-resistant and highly susceptible derivatives of *H. influenzae* HMC (21). They speculated that another pump(s) might be involved in macrolide efflux in *H. influenzae*. Cloning of individual genes *acrA*, *acrB*, and *tolC* from strains with differential efflux-mediated susceptibility to azithromycin and telithromycin and chimeric analysis (not performed in the current study) will be necessary to identify the mechanism of this differential susceptibility.

Very recently, Chollet et al. have demonstrated that in *acrAB* and *tolC* mutants of *Enterobacter aerogenes*, erythromycin MICs decreased 32 times, compared to only 4 times for telithromycin (4). By contrast, phenylalanine arginine β-naphthylamide (PAβN) exposure produced similar 16-fold reduction in both erythromycin and telithromycin MICs. Furthermore, ketolide uptake was significantly increased in the presence of increasing PAβN concentrations. These findings allowed the authors to conclude that in *E. aerogenes* the AcrAB/TolC complex is able to efficiently expel macrolides, such as erythromycin or clarithromycin, while another mechanism that is (PAβN) sensitive but AcrAB/TolC independent pumps out telithromycin. Absence of complete correlation between telithromycin and azithromycin MICs and, most importantly, between the presence of azithromycin and telithromycin efflux in some strains may support involvement of other factors in macrolide and telithromycin efflux. Recently a new macrolide-specific ABC-type efflux transporter has been identified in *E. coli* (13). ATP-binding cassette (ABC) transporters are known to be the major drug efflux pumps in mammalian neoplastic cells and have also been identified in gram-positive bacteria. Kobayashi et al. showed that MacAB complex confers TolC-dependent macrolide (14- and 15-membered) resistance via active drug efflux (13).

In summary, our study indicates that *H. influenzae* strains for which the telithromycin MICs were ≤0.25 μg/ml lack telithromycin efflux, and strains for which the telithromycin were MICs ≥0.5 μg/ml have efflux present. Thus, a telithromycin resistance mechanism (telithromycin efflux pump) is present even among strains that are considered susceptible according to existing CLSI recommendations. The only category of *H. influenzae* strains that seem to have no ketolide resistance mechanisms (neither target alteration nor efflux pumps) is a small group of telithromycin-susceptible *H. influenzae* strains which are also highly susceptible to azithromycin and clarithromycin. Antimicrobial therapy with these compounds may be ineffective, while their extensive use may further select for mutations in ribosomal targets and overexpression of the efflux pump(s). Alternative antimicrobials, such as broad-spectrum quinolones, which are also active against pneumococci, may be necessary to improve the efficacy of antimicrobial chemotherapy of *H. influenzae* infections, especially in older patients with severe acute exacerbations of chronic bronchitis.

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