

INTRODUCTION

Macrolide resistance occurs by:

- Methylation of the 23S rRNA by *erm* genes.
- Mutations in 23S rRNA and riboproteins L4 and L22.
- Active efflux (encoded by the *mef* class of genes)⁽¹⁾.
- The aim of this investigation was to determine the mechanisms of resistance in high-level macrolide resistant *H. influenzae* (HLMR HI) collected as part of the BSAC Survey 2006/2007.

METHODS

- MIC determination in the presence of carbonyl cyanide m-chlorophenylhydrazone (CCCP)(0.06 mg/L) and phenyl-arginine-β-naphthylamide (PABN)(64 mg/L) and genotyping for the presence of the genes *erm* and *mef* were performed according to previously published protocols^(2, 3).
- Amplification of the 6 alleles of HI 23S rRNA was performed by using a modification of a previously published method⁽⁴⁾.
- The common forward primer and allele specific reverse primers are listed in Table 1.
- Long PCR cycling parameters were: 94°C 2 min, (92°C 30 s, 51°C 30 s, 70°C 7 min 30 s) x15, (92°C 30 s, 51°C 30 s, 70°C 7 min 30 s + increase extension time by 15 s

with every successive cycle) x18, 70°C 10 min.

- Nested PCR primers HI_23S_DII_F1 and HI_23S_DII_R1 and Domain V HI_23S_DV_F1 and HF2771 are listed in Table 1.
- Nested PCR cycling parameters were: 94°C 2 min, (92°C 30 s, 55°C 30 s, 72°C 40 s) X25, 72°C 7 min for 23S amplification
- Riboprotein amplification was performed using a modified variation of a previously published method⁽⁵⁾.
- Confirmation of the presence of PCR amplification products were confirmed by agarose gel electrophoresis, using standard procedures.
- Sequencing of PCR products was performed using standard procedures.

Table 1. Primers for the sequencing of the 23S rRNA genes of *H. influenzae*

Description	Primer name	Sequence 5'-3'
Forward (common)	HI_23S_F ⁽⁴⁾	AAG CAA TCA AGT GTT TAG T
Reverse Allele A	HI_23S_A_R4 ⁽⁴⁾	CTG AAG AAG AGG TTT GAC T
Reverse Allele B	HI_23S_B_R4 ⁽⁴⁾	CAA TCG TGC CTC CCT GAA TA
Reverse Allele C	HI_23S_C_R4 ⁽⁴⁾	TTC TGG CGA CAC ATA AAG TAG
Reverse Allele D	HI_23S_D_R4 ⁽⁴⁾	TTA GAT GCA ATG GTG ATA GC
Reverse Allele E	HI_23S_E_R5 ⁽⁴⁾	CAA AAT CCG TAC CTA AAT GAA AC
Reverse Allele F	HI_23S_F_R4 ⁽⁴⁾	CGC AAA CAT CGA GCC AAC CA
Domain II Forward	HI_23S_DII_F1 ⁽⁴⁾	TCA CGC ACT TAT ATT TTG TAG
Domain II Reverse	HI_23S_DII_R1 ⁽⁴⁾	AGC TGG CGG TCT GGG TTG TTT C
Domain V Forward	HI_23S_DV_F1 ⁽⁴⁾	TGG TGT CAT CGA AAG AGA AGC
Domain V Reverse	HF2771 ⁽⁵⁾	CAA GTT TCG TGC TTA GAT G
L4 Forward	HL_L4F ⁽⁵⁾	TTA AGC CGGC AGT TAA AGC
L4 Reverse	HL_L4R ⁽⁵⁾	CAC TTA GCA AAC GTT CTT G
L22 Forward	HL_L22F ⁽⁵⁾	CGG CAG ATA AGA AAG CTA AG
L22 Reverse	HL_L22R ⁽⁵⁾	TGG ATG ACT TTT GAC CC
sequencing primer	HF2330 ⁽⁵⁾	GTA TAA GCA AGC TTA ACT G
sequencing primer	HI_23S_DV_R1 ⁽⁴⁾	CGA TCG CGC ACC GTG ATT AG

(a) primer sequences designed for this study; (5) reference source for primer sequence

•Additional primers for 23S sequencing (HF2330 & HI_23S_DV_R1) (Table 1).

RESULTS

- No known *erm* or *mef* genes were found.
- The effect of efflux inhibitors and nucleotide mutations in domain V of the 23S rRNA and the amino acid changes in the riboproteins are shown in Table 2 (*E. coli* numbering.)

CONCLUSIONS

- HLMR in these isolates of HI was due to active efflux, mutations in riboproteins L4 or L22 and/or the 23S rRNA gene as previously reported ⁽⁵⁾.

CONCLUSIONS (contd)

- Although, similar mutations in 23s and riboproteins do not always result in such high macrolide MIC values.
- We describe a single mutation in 23S rRNA at position 2059 which on its own may confer an ERY MIC of 2048 mg/L (HI8).
- The 8 isolates tested were from separate hospitals and each had disparate resistance mechanisms. This suggests HLMR in HI is not spread as a result of a resistant clone but by separate mutational events.

Table 2 Macrolide specific mutations observed in the 23 rRNA gene and Ribotroteins

	Erythromycin MIC (mg/L)			23S			L4	L22	
	alone	with CCCP	with PABN						
HI1	128	128	32	-	G2160U	G2162A	A2163U	T64K	G91D
HI2	256	32	<1	-	G2160U	G2162A	A2163U	W	W
HI3	256	16	4	-	G2160U	G2162A	A2163U	W	83DelK
HI4	512	64	<1	-	G2160U	G2162A	A2163U	W	95InsRID
HI5	1024	512	16	-	G2160U	G2162A	A2163U	W	90InsN, 103InsH
HI6	1024	1024	256	-	G2160U	G2162A	A2163U	W	W
HI7	2048	1024	128	A2058G	G2160U	G2162A	A2163U	W	W
HI8	2048	512	128	A2059C	-	-	-	W	W

Where: W = Wild Type, Ins = Insertion and Del = Deletion

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Central Laboratory: Quotient Bioresearch Ltd, Cambridge.

Sponsors 1999/2000 - 2006/07: Abbott, Aventis, Bayer, GeneSoft, GSK, MSD, and Wyeth.

Support: BSAC.

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