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昆明市 10 岁以下儿童大环内酯类耐药肺炎支原体情况

王霖[1]，宾松涛[1]，郝芮[1]，吴玉芹[1]，谭力[1]，袁涛[2]，叶冬梅[1]，
胡晓琴[1]，邓东佳[1]，张婷[1]，殷峥[1]，施慧[3]，李明[1]

(1. 昆明市儿童医院呼吸内科，昆明 650000；2. 云南省第一人民医院妇产科，昆明 650034；
3. 中国人民解放军东部战区总医院消化内科，南京 210009)

[摘　要]　**目的：**探讨昆明市10岁以下儿童大环内酯类耐药肺炎支原体感染情况分析。**方法：**收集昆明市儿童医院社区获得性呼吸道感染患儿500例，其中男237例，女263例，年龄3~10岁。采用聚合酶链反应(polymerase chain reaction，PCR)扩增并测序肺炎支原体耐药株的23S rRNA基因靶区V区域。聚类分析用于预测耐药支原体的变异与传播。**结果：**在500例呼吸道感染患儿的痰标本中，成功培养出213株，其中药敏菌株70株，中敏菌株96株，耐药菌株47株，耐药菌株检出率为22.06%。用23S rRNA V区引物进行PCR扩增出47株耐药菌株，8株耐药菌株未能扩增，39个耐药菌株的目标条带成功扩增。测序结果显示：16株菌株与标准菌株M129高度同源，16株耐药菌株中，有4株与标准菌株M129相比无基因位点突变。4株含有A2063G转化，3株含有A2064G转化，5株含有A2064C转化。2064点突变的频率(大肠杆菌中为2059)比2063点突变的频率高得多。人耐药肺炎支原体对5种抗生素的表型耐药模式因突变位置不同而不同。突变株A2063G表现出对红霉素和罗红霉素的抗性，突变株A2064G表现出对红霉素和交叉霉素的抗性，突变株A2064C表现出对红霉素的抗性。**结论：**该研究有助于更好地筛选抗支原体药物，并在临床上更好地治疗对支原体有耐药性的感染患儿。

[关键词]　大环内酯类耐药肺炎；儿童；突变株；聚合酶链反应

Macrolide-resistant *Mycoplasma pneumoniae* in children under 10 years old in Kunming

WANG Lin[1], BIN Songtao[1], HAO Rui[1], WU Yuqin[1], TAN Li[1], YUAN Tao[2], YE Dongmei[1], HU Xiaoqin[1], DENG Dongjia[1], ZHANG Ting[1], YIN Zheng[1], SHI Hui[3], LI Ming[1]

(1. Department of Respiratory Medicine, Kunming Children's Hospital, Kunming 650000; 2. Department of Obstetrics and Gynecology, First People's Hospital of Yunnan Province, Kunming 650034; 3. Department of Gastroenterology, General Hospital of PLA Eastern Theater Command, Nanjing, 210009, China)

Abstract　**Objective:** To investigate the infection of macrolide-resistant *Mycoplasma pneumoniae* in children under 10 years

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通信作者 (Corresponding author)：李明，Email: etyyliming@126.com

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old in Kunming. **Methods:** A total of 500 children with community acquired respiratory tract infection in Kunming Children's Hospital were collected, including 237 boys and 263 girls, aged 3–10 years. The region V of 23S rRNA gene target region of *Mycoplasma pneumoniae* resistant strain was amplified and sequenced by polymerase chain reaction (PCR). Cluster analysis was used to predict the variation and spread of drug-resistant mycoplasma. **Results:** Among 500 sputum samples from children with respiratory tract infection, 213 strains were successfully cultured, including 70 drug-sensitive strains, 96 medium-sensitive strains and 47 drug-resistant strains, and the detection rate of drug-resistant strains was 22.06%. Forty-seven drug-resistant strains were amplified by polymerase chain reaction with 23S rRNA V region primers, 8 drug-resistant strains failed to be amplified, and the target bands of 39 drug-resistant strains were successfully amplified. Sequencing results showed that 16 strains were highly homologous to standard strain M129, and 4 of 16 drug-resistant strains had no gene mutation compared with standard strain M129. There are 4 strains containing A2063G transformation, 3 strains containing A2064G transformation and 5 strains containing A2064C transformation. The frequency of 2064 point mutation (2059 in *E.coli*) was much higher than that of 2063 point mutation. Phenotypic resistance patterns of drug-resistant *Mycoplasma pneumoniae* to five antibiotics were different due to different mutation positions. The mutant A2063G showed resistance to erythromycin and roxithromycin, the mutant A2064G showed resistance to erythromycin and cross-mycin, and the mutant A2064C showed resistance to erythromycin. **Conclusion:** This study is helpful to better screen the anti-mycoplasma drugs and better treat the infected children with mycoplasma resistance in clinic.

Keywords macrolide resistant pneumonia; children; mutant; polymerase chain reaction

肺炎支原体(mycoplasma pneumonia，MP)是社区获得性肺炎的主要原因之一，尤其在年轻人中[1-2]。MP在北美、欧洲、拉丁美洲和亚洲不同地区的检出率分别为22%、28%、21%和20%，成人的检出率为10%~40%[3]。但目前对儿童支原体肺炎的研究不多见。大环内酯类抗生素(macrolides，MLs)是肺炎支原体感染的首选抗生素[4-5]，MLs及其衍生物具有很强的渗透质膜的能力[6-7]，可以通过与大核糖体亚单位结合阻断新生多肽的途径，从而抑制蛋白质合成[8]。长期使用抗生素治疗支原体肺炎，尤其是红霉素，容易导致支原体耐药，从而大大降低临床治疗效果。大环内酯类耐药肺炎支原体(macrolide-resistant MP，MRMP)在亚洲越来越多，其中大环内酯类耐药率已上升至90%[9-10]。基于此，本研究对云南省昆明市肺炎支原体肺炎患儿分离的耐药菌株进行点突变分析，有助于更好地筛选抗支原体药物，并在临床上更好地治疗对支原体有耐药性的感染患儿。

1 对象与方法

1.1 对象

收集昆明市儿童医院社区从2019年3月至2019年8月获得性呼吸道感染患儿500例，其中男生237例，女生263例，年龄3~10岁。痰标本放在1.5 mL无菌试管中[11]。本研究经昆明市儿童医院医学伦理委员会审批，受试患儿家长均知情同意。

1.2 肺炎支原体的培养

从痰液分离支原体感染患儿的肺炎支原体。PPLO培养基和BBLTM支原体基础培养基添加灭活的20%马血清、10%自制酵母提取物、1%葡萄糖、1 000 U/mL青霉素G和0.025%乙酸铊作为液体培养基，液体培养基中加入1.5%琼脂用作制备琼脂培养基。本研究中使用的双相培养基由2 mL液体培养基和1 mL琼脂培养基组成，两者都添加有0.002%酚红和0.001%亚甲蓝。培养基的颜色变化表明肺炎支原体呈阳性。所有培养均在37 ℃有氧条件下进行[12]。

1.3 最小抑菌浓度检测

根据既往研究[13-14]，使用微量肉汤稀释法测量抗生素的最小抑制浓度。测试的抗生素包括阿奇霉素、红霉素、克拉霉素、罗红霉素和交沙霉素。简而言之，将在含有10^4~10^5 CFU/mL肺炎支原体的PPLO培养基中制备的连续2次稀释的抗生素加入96孔微孔板中。当对照培养基的颜色改变时，此时被认为是最低抑菌浓度[15]。最后筛选出不同抗生素对应的耐药菌株。

1.4 聚合酶链反应

将耐药菌株悬浮在少量生理盐水中，混合均匀，以2 000 r/min离心15 min。按照说明书，使用QIA amp DNA试剂盒从上清液中提取DNA。正向引物为5'-GCAGTGAAGAACGAGGGG-3'，反向引物为5'-GTCCTCGCTTCGGTCCTCTCG-3'。聚合酶链反应(polymerase chain reaction，PCR)反应条件：预变性94 ℃ 5 min；变性94 ℃ 30 s，退火55 ℃ 30 s，延伸72 ℃ 1 min，变性、退火、延伸3个步骤重复40个循环；最终延伸72 ℃ 5 min。退火温度根据经验进行了优化，以给出最佳的特异性[16]。PCR产物于1.5%琼脂糖凝胶上进行电泳。PCR条带大小与对照相同的菌株将PCR样本送至测序公司进行测序验证。

1.5 比较基因组

为探索耐药支原体的进化和传播规律，利用MEGA软件对原肺炎支原体与耐药菌株的23S rRNA基因序列进行比较[17]。

1.6 微生物系统发育分析

在利用现代分子生物学技术在分子和基因水平上获得大量的分类单元尤其是种的遗传信息后，来推断和重建微生物类群的演化历史和演化关系，即建立系统发育树，根据分离菌株的16S rDNA或18S rDNA序列与相关微生物种之间的同源性，将分离获得的菌株放置于系统发育树的确当分支位置，以显示其在系统发育中的地位和与其他种间的亲缘关系。

1.7 统计学处理

采用SPSS 21.0统计学软件进行数据分析，使用McNemar's检验(配对χ^2检验)，P<0.05为差异有统计学意义。

2 结果

2.1 213 例痰培养中肺炎支原体的药敏分析

在500例呼吸道感染患儿的痰标本中，成功培养出213株，并对其进行了大环内酯类无内酯类药物(阿奇霉素、红霉素、克拉霉素、罗红霉素、交沙霉素)的药敏试验。按照美国国家临床实验室标准委员会(National Committee for Clinical Laboratory Standards，NCCLS)2001年规定的临界浓度来判断，药敏菌株70株，中敏菌株96株，耐药菌株47株，耐药菌株检出率为22.06%(表1)。

2.2 47 株耐药菌株 23S rRNA V 区 PCR 分析

将扩增产物与标准MP菌株M129(GeenBank: U00089.2)进行比较。8株耐药菌株未能扩增，可能是分析灵敏度有限。39个耐药菌株的目标条带成功扩增，并进行测序(图1)。

参照菌株M129和39株耐药菌株都进行测定23S rRNA基因的V区序列，测序结果显示16株菌株与标准菌株M129高度同源(图2)。进一步分析结果显示：16株耐药菌株中，有4株与标准菌株M129相比无基因位点突变；4株含有A2063G转化，3株含有A2064G转化，5株含有A2064C转化。在本研究中，2064点突变的频率(大肠杆菌中为2059)比2063点突变的频率高得多。人耐药肺炎支原体对5种抗生素的表型耐药模式因突变位置不同而不同。突变株A2063G表现出对红霉素和罗红霉素的抗性，突变株A2064G表现出对红霉素和交叉霉素的抗性，突变株A2064C表现出对红霉素的抗性(表2)。

表1 213例痰培养肺炎支原体药敏结果分析

Table 1 Analysis of drug sensitivity of 213 cases of Mycoplasma pneumoniae cultured in sputum

抗生素	敏感性/株	中等灵敏度/株	耐药/株
阿奇霉素	23	12	8
红霉素	4	23	12
克拉霉素	7	16	10
罗红霉素	4	21	12
交沙霉素	32	24	5
总计/[株(%)]	70 (32.86)	96 (45.07)	47 (22.06)

在当前的研究中，39个耐药菌株的DNA序列被用来构建一个相关连接系统发生树，以表示分离株的基因组相关性(图2)。系统发育分析表明：分离出16株与已知参考菌株M129分类相对应的菌株，它们与M129序列具有96%的核苷酸同源性。

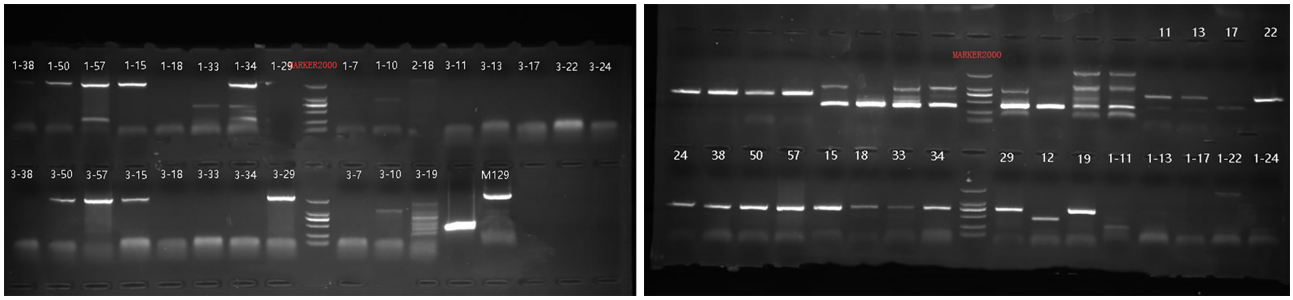

图1 47个菌株23S rRNA V区PCR产物的凝胶电泳图

Figure 1 Gel electrophoresis of PCR products of 23S rRNA V region of 47 strains

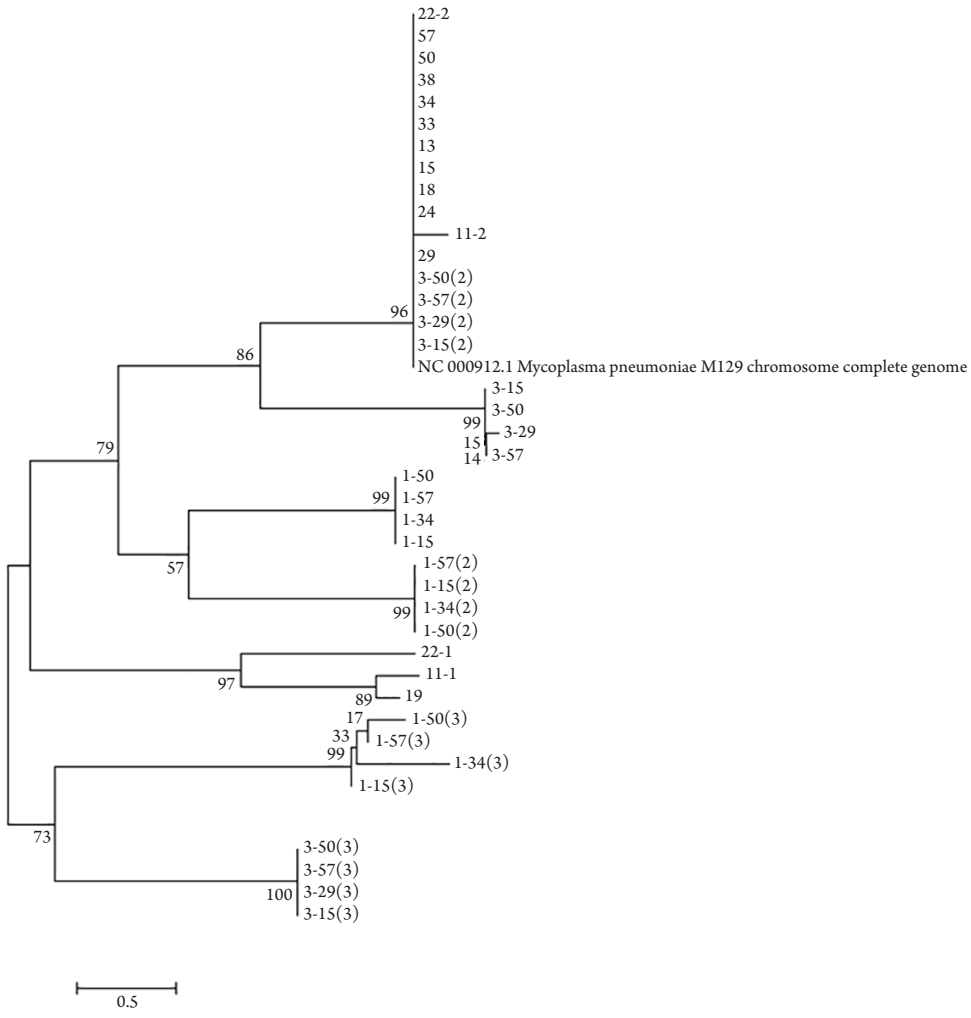

图2 39株耐药菌株23S rRNA序列的进化树分析

Figure 2 Phylogenetic tree analysis of 23S rRNA sequences of 39 drug-resistant strains

表2 16株体外耐药菌株的药物敏感性和突变位点

Table 2 Drug sensitivity and mutation sites of 16 drug-resistant strains in vitro

分离株	突变点	最低抑菌浓度/(μg·mL^{-1})				
		阿奇霉素	红霉素	克拉霉素	罗红霉素	交沙霉素
M129	N/A	0.002	0.039	64	0.5	0.03
4株	N/A	0.002	0.039	64	0.5	0.03
4株	A2063G	0.001	>400	300	400	9.35
3株	A2064G	0.001	300	35	75	400
5株	A2064C	0.001	300	35	95	250

3 讨论

肺炎支原体引起的社区获得性肺炎通常在临床上采用大环内酯类药物治疗[18]。在亚洲，肺炎支原体对大环内酯类抗生素的耐药性正在逐年增加。因此，迫切需要开发一种灵敏、准确的方法来检测肺炎支原体阳性临床标本中的大环内酯类抗生素耐药性[19]。

本研究通过PCR扩增耐药菌株和标准菌株M129的MP抗性基因片段(mp-23s rRNA V区)，结果表明：在47株抗性菌株中，只有39株与标准菌株的电泳条带一致，这可能是系统灵敏性造成的。39株耐药菌株进行测序并与美国国家生物信息中心(National Center for Biotechnology Information，NCBI)检索的MP-23 s rRNA基因序列进行比对，结果显示：有16株与M129 23 s rRNA基因序列同源，有4株序列A2063G突变，有3株A2064G突变，有5株A2064C突变，其余4株无基因突变，可能是其在存在其他抗性机制。A2063G、A2064G和A2064C突变与分离株的大环内酯类耐药相关。在大环内酯类药物治疗期间，从易感基因型发展到耐药基因型的菌株已有报道[20-24]。这一现象强调了检测大环内酯类药物耐药性的快速诊断方法的重要性。

本研究对39株耐药菌株和标准菌株M129的基因序列进行系统发育分析，探讨耐药支原体的进化规律，结果显示：16株菌株与标准菌株M129具有96%的同源性，表明耐药支原体的进化过程中没有出现新的基因组，但是有趣的是，这与先前研究[25]的结果不一致，可能是受区域因素的影响。

昆明市支原体序列类型与标准菌株M129同源，耐药支原体基因突变主要在A2063G、A2064G和A2064C，与以往研究[16]一致。在5种抗大环内酯类药物中，阿奇霉素的最低抑菌浓度较低，提示在临床上可能更倾向于治疗对肺炎支原体有耐药性的感染患者。

综上所述，耐药支原体序列在23S rRNA V区存在突变，耐药肺炎支原体的突变位点主要为2063和2064。根据测序点的突变结果，精确用药不仅可以提高疗效，还可以减少滥用抗生素引起的抗生素耐药问题。

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