·论 著·

利奈唑胺诱导粪肠球菌耐药50S核糖体蛋白突变位点分析

刘晓军[1,2],陈重[1,2],李多云[1,2],程航[1,2],邓向斌[1,2],邓名贵[1,2],潘伟光[1,2],杨唯枝[1,2],王红燕[1,2],
姚伟明[1,2],余治健[1,2],邓启文[1,2*]

1.广东医学院附属深圳市南山区人民医院感染科,广东 深圳 518052;

2.深圳市内源性感染诊治重点实验室,广东 深圳 518052

摘要:目的 阐明体外诱导利奈唑胺耐药粪肠球菌的核糖体蛋白位点变异特征。**方法** 收集1株血流感染的粪肠球菌利奈唑胺敏感株,通过体外浓度倍增法诱导利奈唑胺耐药;挑取单克隆,经E-test条测定MIC值,获得各菌株的耐药浓度梯度;提取耐药菌株基因组DNA,PCR扩增核糖体蛋白L3和L4(对应rplC和rplD基因),扩增产物经测序后与野生株比较,获得核糖体蛋白及对应氨基酸的突变位点。**结果** 经体外多步法诱导利奈唑胺耐药的不同MIC值粪肠球菌共13株。PCR测序分析2株母株均无变异位点,rplC基因对应的氨基酸位点不尽相同,rplD基因普遍存在T301C位点变异,对应的氨基酸为Phe101Leu。**结论** 体外多步法可诱导粪肠球菌利奈唑胺耐药,耐药机制与核糖体蛋白位点突变密切相关,但仍需进一步研究证实突变位点与耐药的关系。

关键词: 粪肠球菌;利奈唑胺耐药;50S核糖体蛋白

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Genetic mutations in 50S ribosomal protein of linezolid-induced drug-resistant *Enterococcus fecalis* in vitro

LIU Xiao-jun[1,2], CHEN Zhong, LI Duo-yun, CHENG Hang, DENG Xiang-bin, DENG Ming-gui, PAN Wei-guang, YANG Wei-zhi, WANG Hong-yan, YAO Wei-ming, YU Zhi-jian, DENG Qi-wen

1.Department o f Infection Diseases in Nanshan District people's Hospital Affiliated to Guangdong Medical College, Shenzhen 518052, , Guangdong, P.R. China; 2.Key Laboratory of New Technology Research on Hospital Infection Diagnosis and Treatment, Shenzhen 518052,Guangdong, P.R. China

Corresponding author:DENG Qi-wen,E-mail:qiwendeng@hotmail.com

Abstract: Objective To investigate the characteristics of genetic mutations in 50S V ribosomal protein gene of linezolid-induced drug-resistant Enterococcus fecalis (*E.fecalis*)in vitro. **Methods** One *E.fecalis* strain numbered as F3 isolated from the clinical sample and the F4, ATCC29212 the most common strain for quality control of *E.fecalis* antibiotic tests, was induced by linezolid with series dilution methods in vitro. Two mother strains, F3 and F4, and series of LZD-resistant ones were isolated and the MIC value was identified by E-Test. Then, the 50S ribosomal protein genes of these strains were amplified and sequenced. **Results** Thirteen linezolid-resistant *E.fecalis* induced in vitro was isolated and identified. Then, PCR and sequencing results indicated the genetic mutations in 50S V ribosomal protein gene of these strains contained multiple point mutations in rplC gene, while in rplD gene, the common mutation was at T301C, and corresponding mutation of amino acid was Phe101Leu. **Conclusion** Linezolid-resistant *E.fecalis* can be induced in vitro. Linezolid resistance in *E. fecalis* was closely associated with the genetic mutations in 50S ribosomal protein genes, but the resistant mechanism needed to be further clarified.

Key words: Enterococcus fecalis; Linezolid resistance; 50S ribosomal protein

利奈唑胺(linezolid,LZD)是一种新型噁唑烷酮类抗菌药物,曾被认为是治疗革兰阳性菌的特效药,因为该药与其他抗菌药物不易产生交叉耐药[1],是目前治疗粪肠球菌医院感染暴发流行的主要药物之一。然而从LZD全球上市开始即不断有临床耐药菌株的报导[2],美国和欧洲各地均有LZD耐药的肠球菌及葡萄球菌出现。随着LZD耐药菌株的不断出现,人们日渐重视对其耐药机制的研究[3]。近年对LZD耐药肠球菌的研究发现,其耐药机制之一是23S rRNA V区基因发生变异,主要是G2576U[4];二是细菌核糖体甲基转移酶基因cfr的获得;第三则是编码细菌50S核糖体蛋白基因rplD和rplC的变异[5-6]。为了进一步探

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作者简介:刘晓军(1974~),男,本科,主治医师,研究方向:细菌耐药机制。

***通讯作者:**邓启文,E-mail:qiwendeng@hotmail.com

讨粪肠球菌50S核糖体蛋白突变位点与LZD耐药的关系，本研究应用LZD体外诱导粪肠球菌耐药，通过基因测序分析，寻找新的50S核糖体蛋白基因变异特点，旨在减少临床耐药菌株的出现。

1 材料与方法

1.1 材料

1.1.1 菌株 粪肠球菌实验菌株由深圳市南山区人民医院提供（编号为F3），该菌株为血液培养阳性的致病菌株。粪肠球菌质控菌株ATCC29212（编号为F4）购自广东省临床检验中心。

1.1.2 试剂和仪器 LZD为美国Pfizer公司产品；LZD E-test条为法国梅里埃公司产品；Mueller-Hinton(MH)琼脂和抗生素敏纸片为英国OXOID公司产品；哥伦比亚血琼脂平板培养基购于广州迪景微生物公司；细菌DNA提取试剂盒QIAamp DNA Mini kit为德国QIAGEN公司产品；PCR反应试剂、DNA Maker（TaKaRa）；DNA片段纯化试剂盒（TaKaRa DNA Fragment Purification Kit Ver.3.0）、T载体（pMD™19-T Vector Cloning Kit）均为日本TaKaRa公司产品。

1.2 方法

1.2.1 药物敏感性试验 将编号为F3和F4的粪肠球菌复苏，采用微量肉汤稀释法通过BD Phoenix™ 100自动细菌鉴定/药敏系统鉴定，根据2012年美国临床和实验标准委员会（CLSI）标准判读结果，测定2株粪肠球菌对LZD的MIC值。

1.2.2 体外多步法诱导LZD耐药 将2株粪肠球菌分别接种到血琼脂平板培养基中复苏。挑取多个单克隆菌落，依次接种到LZD浓度（起始浓度为1μg/mL）倍比增加的M-H琼脂培养基中，37 ℃培养16 h，每个药物浓度培养3～5代，当细菌生长不良时降低药物浓度重复传代培养，每个药物浓度最多培养10代。待LZD浓度为4 μg/mL且反复传代数次后增加LZD浓度（不超过10代），逐渐倍比增加浓度至256μg/mL，筛选阳性克隆并对诱导出的耐药菌用LZD E-test条测定MIC值，建立粪肠球菌对LZD不同MIC值梯度菌株群（包括1~256μg/mL组）。另接种无抗生素平板进行同期培养，观察细菌生长情况。为保证耐药菌株的稳定性，在确定其MIC值之前，应用无抗生素培养基培养3代。

1.2.3 50S核糖体rplC和rplD基因扩增和测序 采用Qiagen试剂盒，根据说明书操作方法提取粪肠球菌基因组DNA，参考文献方法[7-8]，以粪肠球菌V583为参照菌株（GeneBank号：NC_004668.1）。采用PCR扩增各菌株的rplC和rplD基因序列（对应的蛋白为L3和L4），引物序列为RL3- 4F：GTACTCATGACCAAAGGAATCTTAG；RL3- 4R：TTCCATTATGCAAGCAACCTCCTC。反应条件为94℃，4min，“94℃，30ec；55℃，30sec；72℃ 2min”共29循环，72℃ 4min。扩增长度1 292bp。Marker为DL10 000 DNA Marker（TaKaRa公司）。PCR产物采用PCR纯化试剂盒纯化，连接T载体后送华大基因公司测序，测序结果与V583序列进行比对，分析软件为DNAMAN.full.version.v5.2.2，标记变异的核糖体蛋白基因核苷酸及氨基酸位点。

2 结果

2.1 LZD诱导粪肠球菌的耐药性 药敏试验结果显示，粪肠球菌F3和F4株诱导前均对LZD敏感。2株母株菌经过多步法体外LZD诱导后，其MIC值显著增高，得到不同MIC值梯度的菌株，经E-test条鉴定后筛选出13株诱导耐药菌株，见表1。

2.2 基因测序分析 50S核糖体蛋白及基因PCR扩增电泳图谱见图1，耐药位点测序结果见表1。F3株在rplC基因中没有统一的耐药氨基酸位点，存在着Ser19 Pro、Lys 66 G lu、Asn184Ser及Gln 103 Arg等氨基酸位点的变异；在rplD基因中，普遍存在T 301 C位点变异，对应的氨基酸为Phe 101Leu，此外还有Gly 85 Val位点的变异。F4组在rplC基因中普遍存在C 395 A位点变异，对应的氨基酸为Pro 132 His，此外还有Ile 188 Thr、Val107 Ile、Lys 207 Arg位点的变异；rplD基因中，普遍存在T 301 C位点变异，对应的氨基酸为Phe 101 Leu；此外还有Lys 117 Gln位点变异。

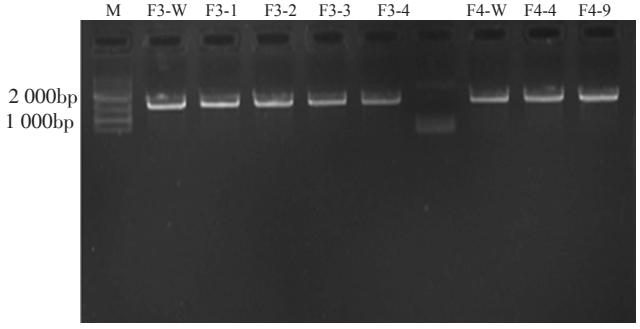

图1 粪肠球菌50S核糖体蛋白基因PCR扩增电泳图

Fig.1 Electrophoretogram of PCR amplification in 50S ribosomal protein of linezolid-induced *Enterococcus fecalis*

3 讨论

利奈唑胺通过与肠球菌23S rRNA V区结合，抑制细菌蛋白合成而产生抗菌作用[9]。随着临床上耐药菌株的不断出现，人们发现利奈唑胺可通过与50S亚基的肽结合位点（PTC）结合，抑制蛋白质的合成从而起到抗菌的作用[10-12]，近年来，已有很多国内学者报导有关革兰阳性球菌利奈唑胺耐药的机制，特别是金黄色葡萄球菌变异位点的报导[13-15]，然而涉及核糖体蛋

表1 粪肠球菌筛选菌株MIC值和核糖体基因位点变异情况

Table 1 MIC values and ribosomal protein gene mutations in selected *Enterococcus fecalis*

菌株 Strains	MIC值(ug/mL) MIC values	核糖体蛋白及氨基酸位点变异检测结果 Results of genetic mutations in ribosomal protein and amino acid			
		rplC	L3	rplD	L4
F3-W*	0.23	W	W	W	W
F3-1	24	T55C	Ser19Pro	G254T	Gly85Val
				T301C	Phe101Leu
F3-2	32	A196G	Lys66Glu	T301C	Phe101Leu
F3-3	64	A551G	Asn184Ser	T301C	Phe101Leu
F3-4	256	A308G	Gln103Arg	T301C	Phe101Leu
F4-W*	0.15	W	W	W	W
F4-1	4	C395A	Pro132His	T301C	Phe101Leu
F4-2	12	W	W	T301C	Phe101Leu
				A349C	Lys117Gln
F4-3	24	T563C	Ile188Thr	T301C	Phe101Leu
F4-4	32	C395A	Pro132His	T301C	Phe101Leu
F4-5	48	C395A	Pro132His	T301C	Phe101Leu
		G319A	Val107Ile		
F4-6	48	C395A	Pro132His	T301C	Phe101Leu
		A620G	Lys207Arg		
F4-7	96	C395A	Pro132His	T301C	Phe101Leu
F4-8	128	C395A	Pro132His	T301C	Phe101Leu
F4-9	256	C395A	Pro132His	T301C	Phe101Leu

注:*表示该菌株系列的野生株(母株);W与野生型比较无位点变异。

Note: *Presented to be wild strain; W:Showed no genetic mutation compared to wild strain.

白耐药相关的利奈唑胺耐药的肠球菌报导相对较少。通过研究50S亚基的晶体结构发现,在靠近23SrRNA的PTC的延伸范围处,存在着很多核糖体蛋白,最关键的一组蛋白就是核糖体蛋白L3,L4及L1。它们虽然不构成PTC的一部分,但其氨基酸残基可以通过与PTC的相互调节作用影响PTC的构象及稳定性[16-17]。L4已被证实与大环内酯类的耐药相关[18-20],去除L4残基Lys68及Gly69的肺炎链球菌导致其对利奈唑胺的敏感性降低,此外,还有一系列已知报导的L4氨基酸位点的变异相继被发现[21-23]。同样的,编码核糖体L3的rplC基因的变异频率也很高,L3蛋白也是和PTC密切作用的核糖体蛋白,但此次实验中,我们在不同的菌株中发现的耐药位点均不相同,有关研究表明,所有关于L3氨基酸位点的变异均集中在一个相同的区域[24-26],基于L3蛋白这一耐药特点,L3蛋白吸收了很多科学家对这一领域的探讨,然而,需要更多的通过直接的定向诱导变异和异源表达的研究来证实L3蛋白氨基酸位点变异与噁唑烷酮类耐药之间的关系。

本研究对诱导耐药的粪肠球菌50S核糖体蛋白基因扫描结果显示,F3和F4的各组菌株之间基因位点变异的差异较大,F3各组菌株在rplC基因中没有统一的位点变异,在rplD基因中,普遍存在Phe101Leu位点。F4各组菌株在rplC基因中普遍存在Pro132His位点变异,在rplD基因中,普遍存在Phe101Leu位点。国内学者研究发现,利奈唑胺耐药的50S核糖体蛋白L3和L4位点的变异与低浓度耐药的肠球菌相关[15],且在本研究中,我们发现高浓度耐药的粪肠球菌仍然存在L3和L4位点的变异,提示C395A(Pro132His)和T301C(Phe101Leu)位点可能是引起耐药的重要位点,仍需通过进一步的蛋白表达功能鉴定。

总体而言,23SrRNA V区核甘酸变异是利奈唑胺耐药发生的主要机制,而核糖体蛋白L3和L4氨基酸变异多与23SrRNA V区变异伴随出现,这些蛋白氨基酸变异可能能够提高细菌的耐药水平,部分细菌甚至对利奈唑胺耐药产生决定性作用,这对指导临床合理用药和减少临床耐药菌株的出现具有一定理论指导意义。

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