淋病奈瑟菌pI优势基因型及其G120/A121突变与耐药性关系

高剑云　陈建忠　赵金方　周坚红

【摘要】　目的　分析浙江省上虞地区淋病奈瑟菌临床菌株外膜孔蛋白优势基因型及其G120和A121突变与耐药性关系。方法　建立能同时检测pIA和pIB基因的双重PCR。目的扩增产物T-A克隆后测序，以分析G120和A121突变情况及证实双重PCR的检测特异性。采用酸性纸片法和二倍琼脂稀释法，分别检测pIA⁺和pIB⁺菌株产β-内酰胺酶情况以及对6种抗生素的耐药性。结果　多重PCR可准确地对所有受检淋病奈瑟菌临床菌株进行porin I(pI)基因分型，其检测灵敏度为1 ng DNA模板。116株淋病奈瑟菌临床菌株中，30.2%(35/116)为pIA⁺菌株，69.8%(81/136)为pIB⁺菌株。所有pIA⁺菌株出现G120D/A121G双突变(88.6%)或A121G单突变(11.4%)，98.8% pIB⁺菌株出现G120K/A121D(65.0%)、G120K/A121G或G120N/A121D(13.8%)双突变以及G120D/N/K单突变(21.3%)。34.5%(40/116)菌株产β-内酰胺酶，其中pIA⁺菌株产酶率(20%)明显低于pIB⁺菌株(40.7%)(P<0.05)。上述临床菌株对青霉素、四环素、环丙沙星和阿奇霉素耐药率高达75.0%～90.5%，仅有3株菌株对头孢曲松耐药，未发现大观霉素耐药菌株。100%和71.4%不产β-内酰胺酶、G120和/或A121突变pIA⁺菌株分别对青霉素和四环素敏感，但不产β-内酰胺酶G120和/或A121突变的pIB⁺菌株对该2种抗生素耐药率均为100%。结论　所建立的多重PCR可用于淋病奈瑟菌pI基因快速和准确分型。上虞地区流行的淋病奈瑟菌主要携带pIB基因。大观霉素和头孢曲松仍可作为治疗淋病的首选药物。G120和/或A121突变增强对青霉素和四环素耐药性仅限于pIB⁺菌株。

【关键词】　淋病奈瑟菌；Porin I基因；双重PCR；耐药性

Correlation between predominant *pI* genotypes, G120/A121 mutations and drug resistance *GAO Jian-yun*[1], *CHEN Jian-zhong*[1], *ZHAO Jin-fang*[2], *ZHOU Jian-hong*[3]. *1 Maternal and Child Health Hospital of Shangyu City, Zhejiang, Shangyu 312300, China; 2 Department of Microbiology and Parasitology, Medical College of Zhejiang University; 3 The Affiliated Gynecological and Obstetric Hospital of Medical College, Zhejiang University*
Corresponding author: ZHOU Jian-hong, Email: Med_bp@zju.edu.cn
【Abstract】　Objective　To analyze the predominant genotypes of outer membrane porin I (PI) and to determine the correlation between G120 as well as A121 mutations in PI proteins and drug resistance in *Neisseria gonorrhoeae* isolates in the local area. Methods　A double PCR to simultaneously detect both *pIA* and *pIB* genes, was established in this study. The target amplification products were T-A cloned and then sequenced to determine the mutations at G120, A121 and the specificity of double PCR. By using acidity slip method and double agar dilution method, the β-lactamase production and resistance to six antibiotics of *pIA*⁺ and *pIB*⁺ gonococcal isolates were detected. Results　Double PCR could be used to accurately genotyping *pI* genes in all the tested gonococcal isolates with the sensitivity of 1 ng DNA template. In the 116 *N. gonorrhoeae* isolates, 30.2%(35/116) were *pIA*⁺ strains and 69.8%(81/136) were *pIB*⁺ strains. All the *pIA*⁺ strains presented G120D/A121G double mutations(88.6%) or A121G single mutation(11.4%). 98.8% of the *pIB*⁺ strains presented G120K/A121D(65.0%), G120K/A121G or G120N/A121D(13.8%) double mutations, and G120D/N/K single mutation(21.3%). 34.5%(40/116) of the isolates produced β-lactamase, and the enzyme-produced rate (20%) in *pIA*⁺ strains was significantly lower than that in *pIB*⁺ strains (40.7%) with P<0.05. No spectinomycin-resistant strains were identified but three ceftriaxone-resistant strains were presented. However, the resistance ratios to penicilin, tetramycin, ciprofloxacin

作者单位:312300 浙江省上虞市妇幼保健院(高剑云、陈建忠)；浙江大学医学院病原生物系(赵金方)；浙江大学医学院附属妇产科医院(周坚红)

通信作者:周坚红,Email:Med_bp@zju.edu.cn

and azithromycin of all the isolates were as high as 75.0%–90.5%. 100% and 71.4% of the *plA*+ strains without β-lactamase production and with G120 and/or A121 mutations were sensitive to penicillin and tetramycin, respectively. On the contrast, 100% of the *plB*+ strains without β-lactamase production and with G120 and/or A121 mutations were resistant to both the two antibiotics. **Conclusion** The established double PCR method could be used for fast and accurate genotyping of *N.gonorrhoeae pl* genes. The *N.gonorrhoeae* strains prevalent in the local areas mainly possessed *plB* gene. Both spectinomycin and ceftriaxone could still be chosen to treat gonorrhea. The resistance enhancement caused by G120 and/or A121 mutations to penicillin and tetramycin was only presented in *plB*+ gonococci.

【Key words】　*Neisseria gonorrhoeae*; Porin I gene; Double polymerase chain reaction; Drug resistanc

淋病奈瑟菌(*Neisseria gonorrhoeae*)感染引起的淋病是我国人群中发病率最高的性传播疾病之一[1-3]。孔蛋白(porin, PI)是淋病奈瑟菌主要外膜蛋白,分为孔蛋白Ⅰ、Ⅱ和Ⅲ(PⅠ、PⅡ和PⅢ)三类,其中60%为PI[4]。PI由一对细菌染色体上等位基因编码,根据其序列及产物免疫原性差异分为PIA和PIB两类,两者氨基酸序列相似性为65%~80%,但一株淋病奈瑟菌或含*plA*基因或含*plB*基因[5]。近年文献报道,不同地区流行的*plA*+和*plB*+淋病奈瑟菌比例有明显差异[6,7],且PIB分子中G120和A121突变与细菌耐药性密切相关[8,9]。为探讨G120和A121突变与细菌耐药性关系,本研究从临床标本中分离了116株淋病奈瑟菌并对其耐药性进行测定,采用能同时检测*plA*和*plB*基因双重PCR对上述菌株进行基因分型,并分析部分*pls*基因扩增产物序列。

材料与方法

1. 菌株来源及培养:采集2008年1—12月在浙江省上虞市妇幼保健院就诊淋病患者泌尿生殖道分泌物标本,接种于含10%脱纤维羊血的GC琼脂平板上(Oxoid),内含VCN抑菌剂和1% ISO-VitaleX增菌剂(Oxoid),36 ℃培养48 h,经菌落形态检查、革兰染色镜检和氧化酶试验确认分离获得116株淋病奈瑟菌。不产β-内酰胺酶淋病奈瑟菌标准株WHO-A、产β-内酰胺酶淋病奈瑟菌WHO-E购自中国药品生物制品检定所,药敏试验质控菌株ATCC49226由浙江大学医学院病原生物学系提供。作为双重PCR特异性检测的大肠埃希菌ATCC25922、金黄色葡萄球菌ATCC25923、铜绿假单胞菌ATCC27543株购自中国药品生物制品检定所,用普通肉汤琼脂(Oxoid)37 ℃培养24 h。

2. DNA提取:收集平板上各细菌新鲜培养物,用生理盐水洗涤并离心沉淀3次,取细菌沉淀用细菌基因组DNA提纯试剂盒(BioColor)提取各菌株基因组DNA。提取的DNA溶于TE缓冲液中,采用紫外分光光度法测定其浓度和纯度[10]。

3. 双重PCR:根据GenBank登录的*plA*和*plB*基因序列及内切酶图谱分析结果,*plA*和*plB*基因5′及3′端序列相同[11,12],故设计一对可扩增全长*pls*基因引物F1和R,由于*plA*基因中间区较*plB*基因短63 bp,且序列相似性较低,故在其中设计仅用于扩增*plB*基因上游引物F2。上游引物F1序列:5′-ATG AAA AAA TCC CTG ATT GCC-3′,上游引物F2序列:5′-GAT ACG GCG AAG GCA CTA AA-3′,下游引物R序列:5′-TTA GAA TTT GTG GCG CAG AAC-3′。引物由上海英骏生物技术有限公司(Invitrogen)合成。PCR总体积为100 μl,内含:2.5 mol/L各dNTP、250 nmol/L各引物、2.5 U Taq-Pfu聚合酶(TaKaRa)、100 ng DNA模板、1×PCR缓冲液(pH值8.3)。PCR参数:94 ℃ 5 min; 94 ℃ 30 s、54 ℃ 30 s, 72 ℃ 90 s, 30个循环; 72 ℃ 10 min。采用溴化乙锭预染1.5%琼脂糖凝胶电泳检测扩增产物。全长*plA*和*plB*基因扩增片段大小分别为984 bp和1047 bp,其中*plB*基因另有一条201 bp扩增条带。

4. 双重PCR灵敏度和特异性检测:从10株淋病奈瑟菌临床分离株、大肠埃希菌ATCC25922、金黄色葡萄球菌ATCC25923和铜绿假单胞菌ATCC27543株提取的基因组DNA采用紫外分光光度法测定浓度[10],然后用TE缓冲液做10倍连续稀释(0.001~100 ng/μl)。在上述多重PCR系统中分别加入不同浓度各菌株DNA模板10 μl,扩增参数及结果观察同上。

5. 序列分析:双重PCR产物经琼脂糖凝胶电泳检测后,采用PCR产物纯化试剂盒(BioColor)回收全长*plA*或*plB*基因扩增片段,提纯的PCR产物质量经紫外分光光度法检测合格($1.8 < A_{260}/A_{280} < 2.1$)后委托Invitrogen公司测序,以了解G120和A121突变情况并考核双重PCR检测结果的准确性。

6. 药敏试验:青霉素、四环素、头孢曲松、大观霉素、环丙沙星、阿奇霉素购自中国药品生物制品检定

所,采用WHO西太平洋地区淋病奈瑟菌耐药监测规划推荐的二倍琼脂稀释法测定各抗生素最低抑菌浓度(MIC)[13]。先将1 ml不同浓度各抗生素溶液加入平皿,GC琼脂加热溶解后冷却至约50 ℃并加入10%脱纤维羊血后倾注平板,使平板中各抗生素浓度均分别为0.015~32 mg/L,37 ℃孵育24 h检菌。各淋病奈瑟菌临床菌株和WHO标准菌株在GC培养基上传代2次后,用24 h新鲜培养物制成0.5麦氏浓度的菌悬液,用多点接种器接种于检菌合格的药敏平板,经36 ℃培养48 h后观察结果,以细菌生长被抑制的药物最高稀释度为MIC。对青霉素、四环素、头孢曲松和大观霉素耐药的判断标准均为MIC≥2 mg/L,对环丙沙星和阿奇霉素耐药的判断标准为MIC≥1 mg/L[13]。

7. β-内酰胺酶检测:采用纸片酸度法测定淋病奈瑟菌株产β-内酰胺酶情况[13]。先将滤纸片用含溴甲酚紫指示剂的青霉素溶液饱和,取待检定淋病奈瑟菌株5~8个菌落涂布于滤纸片上形成直径5 mm菌膜,37 ℃孵育10 min,滤纸片由紫变黄者为β-内酰胺酶阳性,不变色者为阴性。实验中分别以标准菌株WHO-E和WHO-A分别作为阳性和阴性对照。

结　果

1. 双重PCR检测灵敏和特异性:该多重PCR扩增灵敏度为1 ng淋病奈瑟菌DNA,对不同浓度的金黄色葡萄球菌、大肠埃希菌和铜绿假单胞菌DNA扩增结果均为阴性。

2. 双重PCR的pIs分型:116株淋病奈瑟菌临床菌株基因组DNA中均可扩增出预期大小的全长pIA基因或pIB基因片段(图1);其中pIA+菌株35株(30.2%),pIB+菌株81株(69.8%)。双重PCR的pI基因分型结果与测序结果完全相符。

3. β-内酰胺酶检测:在116株淋病奈瑟菌临床菌株中,34.5%(40/116)产生β-内酰胺酶,65.5%(76/116)β-内酰胺酶检测结果阴性。其中35株pIA+菌
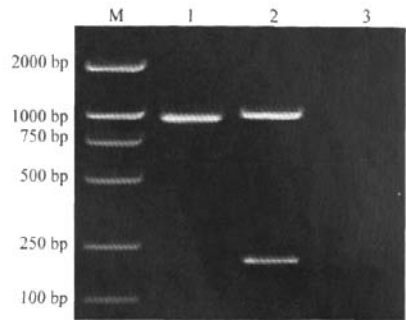
注:M:DNA Marker(BioColor);1、2:从淋病奈瑟菌临床株中扩增的pIA基因片段(984 bp)和pIB基因片段(1047和201 bp);3:空白对照

图1　双重PCR扩增的淋病奈瑟菌pIA和pIB基因片段

和81株pIB+菌中分别有7株(22.8%,7/35)和33株(39.5%,33/81)β-内酰胺酶检测结果阳性,差异有统计学意义(χ²=4.65,P<0.05)。

4. G120和A121突变率及突变类型(表1):所有35株pIA+淋病奈瑟菌临床菌株PIA序列中G120和/或A121均发生突变,但突变形式仅有G120D/A121G双突变(88.6%,31/35)和A121G单突变(11.4%,4/35)两类。pIB+淋病奈瑟菌临床菌株中仅有1株未发生G120和A121突变外,其余80株(98.8%,80/81)均发生G120和/或A121突变且形式多样,但以G120K/A121D双突变为主(65.0%,52/80),仅13.8%(11/80)菌株G120K/A121G或G120N/A121D双突变,21.3%(17/80)菌株在G120位点发生单突变。

5. MIC检测:结果见表2。116株淋病奈瑟菌临床菌株对青霉素、四环素、头孢曲松、环丙沙星和阿奇霉素的耐药率分别为75.0%(87/116)、77.6%(90/116)、2.6%(3/116)、90.5%(105/116)和88.8%(103/116),但未发现耐大观霉素菌株。尽管所有pIA+菌株均有G120和/或A121突变,但其中不产β-内酰胺酶pIA+菌株均对青霉素敏感,71.4%(20/28)不产β-内酰胺酶pIA+菌株对四环素敏感。值得注意的是,本研究中出现3株对头孢曲松中度耐药(MIC=2~4 mg/L)的pIB+菌株。

表1　115株淋病奈瑟菌pIA+或pIB+株的G120和A121突变类型

菌株	β-内酰胺酶	株数	突变形式							
			G120K/A121D	G120K/A121G	G120N/A121D	G120D/A121G	G120D	G120K	G120N	A121G
pIA+	+	7	0	0	0	6	0	0	0	1
	−	28	0	0	0	25	0	0	0	3
pIB+	+	33	22	3	1	0	4	1	2	0
	−	47*	30	5	2	0	6	2	2	0
合计		115	52	8	3	31	10	3	4	4

注:*1株不产β-内酰胺酶pIB+菌株G120和A121未突变

表2 116株淋病奈瑟菌临床菌株药敏试验结果

菌株	β-内酰胺酶	株数	MIC值(mg/L)											
			青霉素		四环素		头孢曲松		大观霉素		环丙沙星		阿奇霉素	
			<2	≥2	<2	≥2	<2	≥2	<2	≥2	<1	≥1	<1	≥1
pIA^+	+	7	0	7	5	2	7	0	7	0	1	6	1	6
	−	28	28	0	20	8	28	0	28	0	2	26	3	25
pIB^+	+	33	0	33	0	33	32	1	33	0	3	30	3	30
	−	48	1°	47	1°	47	46	2	48	0	5	43	6	42
合计		116	29	87	26	90	113	3	116	0	11	105	13	103

注:°同表1

讨 论

淋病奈瑟菌对青霉素和四环素耐药机制主要有质粒介导和染色体介导两大类[3,9]。以往认为淋病奈瑟菌可由质粒基因编码产生β-内酰胺酶,从而导致高水平的质粒介导特异性耐药性[13,14]。近年文献报道,外膜通透性降低也是淋病奈瑟菌对青霉素和四环素耐药有关,可导致中等水平的染色体介导非特异性耐药性[12,13]。由于淋病奈瑟菌具有质粒和/或染色体介导两种耐药机制,从而表现为淋病奈瑟菌菌株的高耐药率[3,9,13]。

PI是具有重要功能的淋病奈瑟菌外膜蛋白,有PIA和PIB两个亚型,不仅与细菌播散性和感染范围相关,也可刺激机体产生具有激活补体、促进吞噬等免疫调理作用的特异性抗体,因而也被认为是制备诊断试剂盒或基因工程疫苗重要的候选抗原[15,16]。已有报道不同亚型淋病奈瑟菌的致病机制有差异[12,14]。此外,G120和A121构成淋病奈瑟菌PIB分子中第三环(loop 3)上2个氨基酸,若编码PIB的pIB基因的G120和A121突变可使PIB分子空间构型发生较大改变,结果使膜通透性显著降低,进入细菌的抗生素明显减少[9,14]。因此,了解不同地区淋病奈瑟菌临床菌株PI基因型及其G120和A121突变与耐药性关系具有现实意义。

全长pIA和pIB基因扩增片段分别为984 bp和1047 bp,其大小十分接近,不易用琼脂糖凝胶电泳加以区分。为了较为方便地对淋病奈瑟菌PI基因分型,我们在分析淋病奈瑟菌株pI基因序列保守区和高变区的基础上,另设计引物用于扩增pIB基因中特有的一个201 bp片段,该201 bp条带可明确指示pIB基因扩增产物。实验结果表明,我们建立的双重PCR能将所有受检的115株淋病奈瑟菌进行pIA和pIB基因分型,对泌尿道常见病原菌金黄色葡萄球菌、大肠埃希菌和铜绿假单胞菌基因组DNA无非特异性扩增,检测灵敏度也可达1 ng淋病奈瑟菌DNA模板,测序结果证实双重PCR分型结果完全正确,表明本研究中建立的双重PCR可快速、简便、敏感、特异地对pI基因准确分型。双重PCR结果显示,116株淋病奈瑟菌临床菌株中,30.2%(35/116)为pIA⁺菌株,69.8%(81/136)为pIB⁺菌株,此分型结果与国外文献报道接近[6,7,9]。

Olesky等[17]采用定位突变技术,分别用D/K/R/P替换淋病奈瑟菌PIB分子中G120、用D/H/P替换A121,结果显示突变菌株增强了对青霉素和四环素耐药性。本次实验结果显示,在81株pIB⁺淋病奈瑟菌临床菌株中,除1株未发生G120和A121突变外,98.8%菌株出现多种形式的G120和/或A121突变,其中65.0%为G120K/A121D双突变、13.8%为G120K/A121G或G120N/A121D双突变,合计双突变率为78.8%,仅21.3%菌株在G120发生单位点突变,未发现独立的A121突变,且G120突变率(100%,80/81)高于A121(77.8%,63/81)。实验中还发现,PIB分子中G120替换的氨基酸按频率高低依次为K/D/N,A121则依次为D/G,为发现G120R/P和A121H/P突变,表明G120和A121自然突变所替换的氨基酸与实验室人工干预有明显差异。此外,所有受检的35株pIA⁺淋病奈瑟菌临床株PIA序列中G120和/或A121均发生突变,但突变形式较PIB简单,仅有G120D/A121G双突变(88.6%)和A121G单突变(11.4%)两类。

本次β-内酰胺酶检测结果显示,仅有34.5%(40/116)淋病奈瑟菌临床株产生β-内酰胺酶,低于国内同类研究报道的45%~47.5%阳性率[18,19]。令人感兴趣的是,40株产β-内酰胺酶菌株中,pIA⁺菌株仅有7株,其余33株均为pIB⁺菌株,其差异有统计学意义(P<0.05)。MIC检测结果显示,淋病奈瑟菌临床株对青霉素、四环素、环丙沙星和阿奇霉素均有很高的耐药率(75.0%~90.5%),但对头孢曲松耐药率仅为2.6%且呈中度耐药(MIC=2~4 mg/L),未发现耐大观霉素菌株,这与国内同类研究报道发现大观霉素耐药株而无头孢曲松耐药株有所不同[18,19]。值得注意的是,尽管所有pIA⁺菌株均有G120和/或A121突

变,其中不产β-内酰胺酶 *pIA*[+] 菌株均对青霉素敏感, 71.4%不产β-内酰胺酶 *pIA*[+] 菌株对四环素敏感,但不产β-内酰胺酶 G120 和/或 A121 突变的 *pIB*[+] 菌株对青霉素和四环素耐药率均为 100%,提示 G120 和/或 A121 突变增强对青霉素和四环素耐药性仅限于 *pIB*[+] 菌株,这与国外文献报道相似[17,20]。

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