·临床研究·

基于全基因组测序的院内耐碳青霉烯肺炎克雷伯菌耐药性及同源性分析

余 清[1],徐 敏[1],史桂兰[1],曹慧玲[1],李 岷[1],吴 倩[2],赵苏瑛[1*]

[1]南京中医药大学附属医院检验科,江苏 南京 210029;[2]南京医科大学公共卫生学院,江苏 南京 211166

[摘 要] **目的**:基于基因组学研究医院内碳青霉烯耐药肺炎克雷伯菌的耐药基因分布,毒力情况及同源性分析,深入阐明院内感染的分子机制,为多重耐药菌株临床治疗和预防提供实验室依据。**方法**:收集1株临床分离的碳青霉烯耐药的泛耐药肺炎克雷伯菌,经培养鉴定,测定对常用抗生素的最低抑菌浓度(minimum inhibitory concentration,MIC),多位点序列分型(multilocus sequence typing,MLST)进行基因分型并行全基因组测序。根据基因测序结果,选择相关耐药基因在临床收集的另外50株碳青霉烯耐药的肺炎克雷伯菌中进行扩增检测,验证其携带情况,对测序结果进行进一步的阐述。**结果**:药敏结果显示该菌对除粘菌素和替加环素外的抗生素全部耐药,测序结果显示该菌染色体基因序列全长5 468 925 bp,含有4个质粒(179 972 bp、141 377 bp、85 181 bp和20 247 bp),共有5 984个蛋白质编码基因,85个tRNA基因和25个rRNA操纵子。此外,该菌携带有大量参与编码多种抗生素的耐药基因。MLST结果显示,该菌基因型为ST11型,该菌大部分序列与之前在四川和杭州报道的菌株类似,最接近的是一株编号SCKP020029(NCBI Accession number:CP029384)的肺炎克雷伯菌。收集的另外50株碳青霉烯耐药的肺炎克雷伯菌确认均为产超广谱β内酰胺酶(extented-spectrum beta-bactamases,ESBLs),其中97%为ST11型。脉冲场凝胶电泳(pulsed field gel electrophoresis,PFGE)结果显示分属3个克隆。在这些菌株中,耐药基因检出率分别为KPC-2(98%),SHV-11(98%),TEM-1(76%),CTX-M(76%),Oqxb1(66%),qnrS(70%),Int1(42%),sul1(82%),sul2(96%),iutA(88%),iucABCD(10%)和rmpA2(100%)。**结论**:实验结果揭示了院内碳青霉烯类耐药肺炎克雷伯菌的基因组学具有高度相似性,流行病学具有聚集性和散发性交叉的特点。抗生素耐药结果提示了细菌耐药中选择性抗生素耐药压力的作用效应,同时质粒耐药基因在细菌中的传播提示这些耐药菌在院内的传播以质粒传播为主,有效的院内感染监测、隔离和控制这类质粒的传播是减少这种细菌耐药感染必不可少的措施。

[关键词] 碳青霉烯耐药;肺炎克雷伯菌;基因组;测序

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Analysis of carbapenem - resistant and homology of hospital - acquired Klebsiella pneumoniae based on whole-genome sequencing

YU Qing[1],XU Min[1],SHI Guilan[1],CAO Huiling[1],LI Min[1],WU Qian[2],ZHAO Suying[1*]

[1]*Department of Laboratory Medicine, Jiangsu Province Hospital of Chinese Medicine, Affiliated Hospital of Nanjing University of Chinese Medicine, Nanjing, 210029*; [2]*School of Public Health, Nanjing Medical University, Nanjing, 211166, China*

[**Abstract**] **Objective**: Based on genomics, this study aims to investigate the distribution of drug-resistant genes, virulence factors, and homology analysis of the hospital isolated carbapenem resistant *Klebislla. pneumoniae* strains. The molecular mechanisms of nosocomial infections will be elucidated to provide laboratory evidence for the clinical treatment and prevention of multidrug-resistant strains. **Methods**: A clinical isolate of carbapenem-resistant pan-drug-resistant *Klebislla. pneumoniae*(KPX)was collected, cultured, and identified. The minimum inhibitory concentrations(MICs)of commonly used antibiotics were determined, and multilocus sequence typing(MLST)was performed for genetic typing, followed by whole-genome sequencing. Based on the results of the genetic sequencing,

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*通信作者(Corresponding author),E-mail:suzan795@163.com

selected relevant resistance genes for amplification detection in an additional 50 clinical isolates of carbapenem-resistant KPX, verified their carriage status, and further elaborate on the sequencing results. **Results**: The drug sensitivity test showed that the KPX strain was resistant to all antibiotics except colistin and tigecycline. The sequencing results relevant that the chromosomal gene sequence of this bacterium is 5 468 925 bp in length and contains four plasmids (179 972 bp, 141 377 bp, 85 181 bp, and 20 247 bp). It had a total of 5 984 protein-coding genes, 85 tRNA genes, and 25 rRNA operons. Additionally, this bacterium carried a large number of resistant genes involved in encoding multiple antibiotics. MLST results indicated that the genetic type of this bacterium is ST11, and most of its sequences are similar to previously reported strains in Sichuan and Hangzhou. The closest match was a strain with the code SCKP020029 (NCBI accession number: CP029384) of *Klebislla. pneumoniae*. The additional 50 carbapenem - resistant *Klebislla. pneumoniae* collected were confirmed to produce extended-spectrum beta-lactamases (ESBLs), with 97% being of ST11. Pulsed-field gel electrophoresis (PFGE) results showed that they belonged to three different clones. Among these strains, the detection rates of resistance genes were as follows: *KPC*-2 (98%), *SHV*-11 (98%), *TEM*-1 (76%), *CTX-M* (76%), *Oqxb*1 (66%), *qnrS* (70%), *Int*1 (42%), *sul*1 (82%), *sul*2 (96%), *iutA* (88%), *iucABCD* (10%), and *rmpA*2 (100%). **Conclusion**: The experimental results revealed that a high degree of genomic similarity among nosocomial carbapenem resistant *Klebislla. Pneumoniae* strains, with epidemiological characteristics of both clustering and sporadic cross-infection. Antimicrobial-resistance profiles suggested the presence of significant selective antibiotic pressure. Furthermore, the dissemination of plasmid-mediated resistance genes among bacteria indicates that the spread of these resistant strains within the hospital is primarily through plasmid transfer. Effective monitoring, isolation, and control of these plasmids are essential measures to reduce the spread of bacterial resistance in nosocomial infections.

[**Key words**] carbapenem resistant; *Klebislla. Pneumoniae*; genome; sequencing

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肺炎克雷伯菌(*Klebsiella pneumonia*, KPN)是临床常见的细菌,常引起原发性肺炎、支气管炎、泌尿道感染。近年来,由于抗菌药物选择压力加大,临床滥用药物情况严重,致使多重耐药肺炎克雷伯菌分离率明显增加。最近十年,肺炎克雷伯菌成为"超级耐药"细菌并史无前例地迅速在全球传播,分离率仅次于大肠杆菌,排在第二位[1]。目前临床上在常规药物无效的前提下都普遍认为碳青霉烯类抗生素是治疗多重耐药肠杆菌科最有效的药物,但是随着碳青霉烯耐药肠杆菌(*carbapenem-resistant Enterobacterales*, CRE)菌株的出现,尤其呈广泛耐药(extensively-drug resistant, XDR)或泛耐药(pandrug-resistant, PDR)特征的菌株检出率的逐年上升,导致临床治疗陷入无药可用的困境。中国CRE监测网数据显示CRE的粗死亡率为33.5%,跟美国数据接近[2]。CRE几乎同时对所有抗生素耐药,因此,对产碳青酶烯酶的多重耐药菌株进行研究显得尤为必要。

泛耐药肺炎克雷伯菌是指对除粘菌素和替加环素外全部抗生素均耐药的菌株,这类菌株在临床上越来越多见,据报道产肺炎克雷伯菌碳青霉烯酶(Klebsiella pneumonia carbapenemase, KPC酶)和新德里酶(New Delhi metallo-β-lactamase, NDM酶)是碳青霉烯耐药的常见原因,但不局限于此[3]。因此本实验中选择1株临床分离的泛耐药肺炎克雷伯菌(KPX),进行全基因组测序,根据测序结果在50株碳青霉烯耐药的肺炎克雷伯菌中进一步进行基因测定和分型。

1 材料和方法

1.1 材料

所有菌株均分离自临床标本,其中用于全基因组测序的菌株KPX分离自1例80岁老年男性的痰液标本,该患者有长期住院史,患有严重的呼吸道疾病。其余的50株碳青霉烯耐药的肺炎克雷伯菌株均来源于院内住院患者,年龄47~98岁,平均年龄78.5岁,男女比2.1∶1。所有试验菌株挑取单个菌落接种哥伦比亚琼脂37 ℃培养过夜,长期保存则置于-80 ℃,使用前复苏。

细菌的鉴定和药敏均使用法国梅里埃VITEK-2 COMPACT全自动系统,结果的判读参照CLSI-M100文件[4]。

1.2 方法

1.2.1 全基因组测序

对菌株KPX的全基因组DNA进行测序。首先使用SOAPdenovo v2.04预先组装Illumina测序数据,然后使用blasR与Pacbio测序数据,使用序列之间的overlap关系连接scaffold,使用CeleraAssembler 8.0软件进行连接组装。在所有scaffold连接完成后,

Illumina数据再次用于验证,最后使用GapCloser v1.12软件用于gap closing的工作。全基因组测序委托上海美吉生物医药科技有限公司进行。

1.2.2　多位点序列分型(multilocus sequence typing,MLST)和脉冲凝胶电泳(pulse-field gel electrophoresis,PFGE)分型

采用7对管家基因对细菌DNA进行PCR扩增,然后对扩增产物进行序列测定,将测许结果上传至数据库,与数据库比对后获取序列分型(sequence type,ST)。

PFGE分型操作为先提取细菌基因组DNA后再用限制性内切酶*Xba* Ⅰ(日本TaKaRa公司)对菌株基因组进行酶切,酶切片断在特定的电泳系统中通过电场方向不断交替变换,电泳15 h后充分分离,根据电泳条带的位置和大小对细菌进行分型。PFGE分型的判断参照Tenover等[5]提出的标准。

1.2.3　耐药基因及毒力相关基因的PCR扩增

使用试剂盒提取细菌DNA和质粒DNA,根据全基因组测序结果选择相关的耐药基因和毒力基因,在临床泛耐药菌株中进行PCR扩增,扩增产物行1.2%琼脂糖凝胶电泳,验证携带情况。选择的基因如表1所示。

2　结　果

2.1　全基因组测序结果

KPX全基因组测序全长5 468 925 bp,环状DNA,含有4个质粒(179 972 bp、141 377 bp、85 181 bp和20 247 bp),其中编码蛋白质的基因5 984个,tRNA基因85个,rRNA操纵子25个。测序结果显示KPX携带多种抗生素耐药基因,包括aadA2、rmtB、blaCTX-M-65、blaKPC-2、blaTEM-1、blaSHV-64、blaSHV-11、qnrS1、carA、sul1、sul2和cat Ⅱ等。这些基因分别能够介导细菌对氨基糖苷类、β内酰胺类、氟喹诺酮类、大环内酯类、磺胺类和氯霉素的耐药。此外,

表1　本实验中所用引物

Table 1　Primers used in this study

基因名称	序列(5′-3′)	片断长度(bp)
rmpA2_F	AGAGTATTGGTTGATAGCCGGA	159
rmpA2_R	GAAATGTCAAGCCACATCCATTG	
iucABCD_F	CCAACTCCGTCCGTACCCTGTCA	838
iucABCD_R	CGAGGGATCGACGATGGTGTTCT	
iutA_F	AATCACCTGGGGGCTGGATGCT	683
iutA_R	CCGCACCTTCCACGCCGTAAAT	
KPC-2_F	GCTACACCTAGCTCCACCTTC	990
KPC-2_R	ACAGTGGTTGGTAATCCATGC	
NDM-1_F	GAAGCTGAGCACCGCATTAG	769
NDM-1_R	GGGCCGTATGAGTGATTGC	
TEM-1_F	ACAGCGGTAAGATCCTTGAGAG	461
TEM-1_R	GAAGCTAGAGTAAGTAGTTCG	
SHV-11_F	ACCTTTAAAGTAGTGCTCTGC	432
SHV-11_R	CACCATCCACTGCAGCAGCTG	
CTX-M_F	ATGGTTAAAAAATCACTGCGTCACTGCGYCAGTTC	876
CTX-M_R	TCACAAACCGTYGGTGACGATTTTAGCCGC	
qnrS_F	ACGACATTCGTCAACTGCAA	417
qnrS_R	TAAATTGGCACCCTGTAGGC	
int1_F	CAGTGGACATAAGCCTGTTC	161
int1_R	CCCGAGGCATAGACTGTA	
sul1_F	CGGCGTGGGCTACCTGAACG	433
sul1_D	GCCGATCGCGTGAAGTTCCG	
sul2_F	GCGCTCAAGGCAGATGGCATT	289
sul2_D	GCGTTTGATACCGGCACCCGT	
oqxB_F	CTGGATTTTCCGTCCGTTTAAC	68
oqxB_R	TTGCCTACCAGTCCCTGATAGC	

在喹诺酮耐药编码区(quinolone resistance-determining region,QRDR)发现了基因的突变,检出了外排泵基因(acrA、acrB、marA、soxS和acrAB)的过表达。基因intI1、aacA4、cmlA1、qacEΔ1、tnpA和blaCTX-M-19都位于Ⅰ类整合子上,2个IS26插入元件之间的序列上携带aadA和sul1基因。此外,还检出了其他一些毒力相关基因,如编码厌氧菌铁载体受体和耶尔森菌素的基因iutA、irp1/2和YbtU/Q/T。MLST分型显示KPX为ST11,与库内相似菌株的亲缘关系如图1所示,大部分类似株来源于中国四川省和浙江省杭州市[6-7],说明国内不同省份流行的泛耐药株局部存在相似性又存在差异性。最相近的一株为SCKP020029(NCBI序列号CP029384),分离自四川某患者[6](图2)。

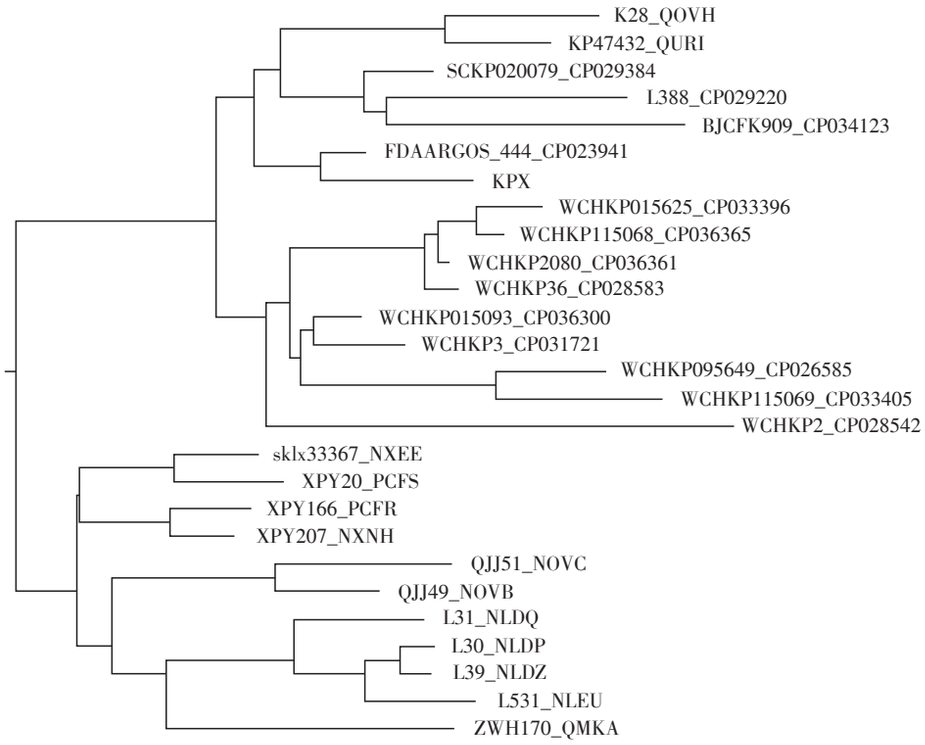

图1　试验菌株与其他菌株的亲缘性分析(SNP)

Figure 1　Analysis of SNP between KPX and other strains

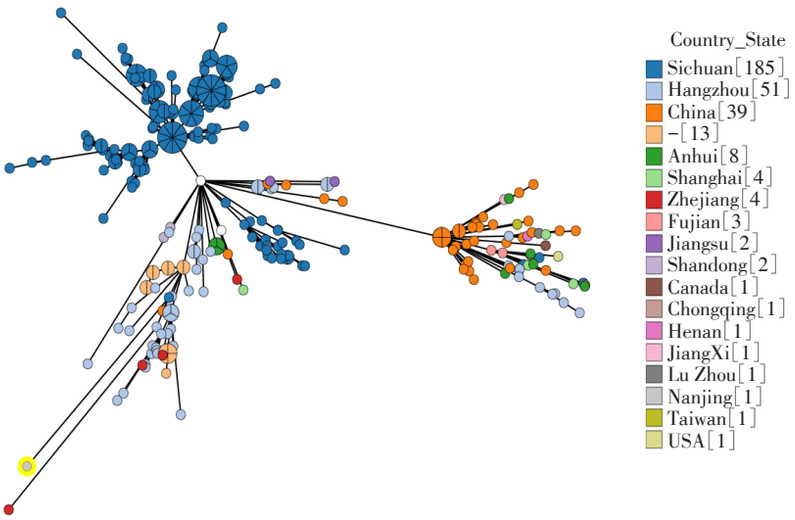

图2　KPX与相似ST11菌株的系统发育关系

Figure 2　Phylogenetic relationship between KPX and similar ST11 strains

KPX的质粒pKPX-A(179 972 bp)上携带一系列毒力基因，包括iucBCD、iutA、rmpA和rmpA2，属于IncFIB/IncHI1B质粒。序列比较后揭示其可能为两个毒力质粒之一，分别为pVir_020079(99.99%)和pKPN-QL24(99.01%)(图3)。质粒pKPX-B(141 377 bp)属于IncFⅡ/IncR质粒，上面携带有碳青霉烯耐药基因blaKPC-2，与分离自四川的产KPC-2肺炎克雷伯菌株SCKP02007中的质粒序列pKPC2_0200799有99.88%的相似度。质粒pKPX-B上的耐药基因盒组合方式为IS26-rmtB-blaTEM-1-IS26、IS26-blaCTX-M-65-IS26-Tn3和IS26-blaSHV-64-blaKPC-2-IS26。此外，IncFⅡ型质粒(85 181 bp)携带有大量的抗生素耐药基因，包括sul2、qnrS1、catⅡ和tet(R/G)，分别介导细菌对磺胺类、氟喹诺酮类、氯霉素类和四环素类药物的耐药[8-12]。

2.2　药物敏感试验结果

50株碳青霉烯耐药的肺炎克雷伯菌药物敏感试验结果见图4。除哌拉西林/他唑巴坦(97.96%)、

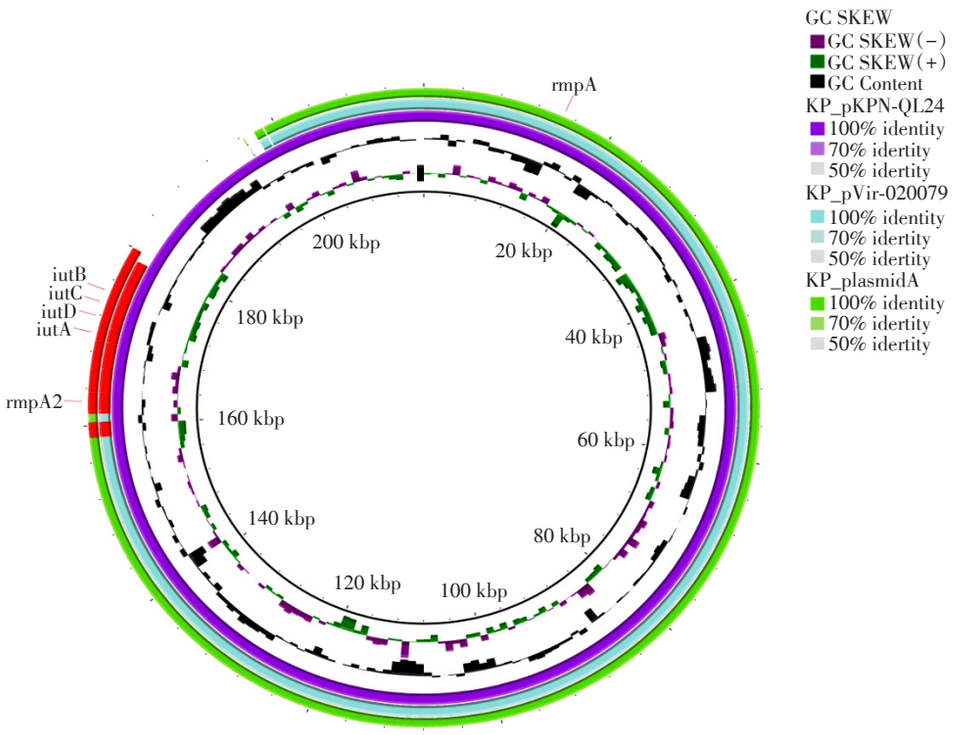

图3　KPX质粒及毒力基因分布图

Figure 3　Distribution of KPX plasmid and virulence genes

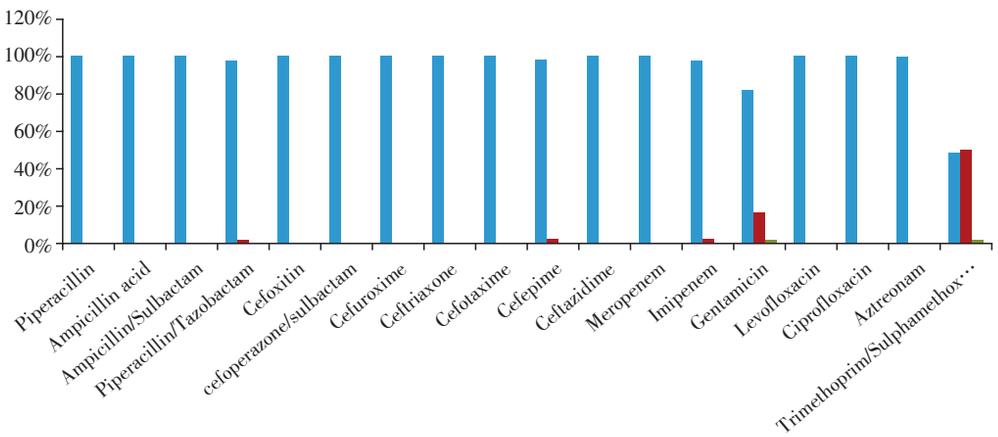

图4　碳青霉烯类耐药肺炎克雷伯菌对常用药物的敏感率(蓝色表示耐药，红色表示敏感)

Figure 4　Sensitivity rate of carbapenem resistant Klebsiella pneumoniae to commonly used drugs(blue indicates resistance, red indicates sensitivity)

头孢吡肟(97.87%)、庆大霉素(81.63%)、复方新诺明(47.92%)外,其余所有药物均100%耐药。

2.3　MLST分型及PFGE分型结果

MLST分型共测出4个型别,分别为ST1(1株)、ST11(47株)、ST15(1株)和ST258(1株),可见绝大部分菌株均为ST11型,与之前报道结果相似。ST258是主要在美国流行的型别[13],这次我们在标本中也检出1株。本质上,ST11是ST258点突变之后形成的。

PFGE结果显示,50株肺炎克雷伯菌酶切条带比较分散,占比较多的主要分属3个带型,其中A带型12株,B带型9株,C带型7株,其余都不同源。带型A、B、C主要分布在重症监护室和急诊病区重症监护室,说明只在小范围内流行并未发生院内大面积传播(图5)。从MLST和PFGE的分型结果看,MLST虽然目前的应用也十分广泛,但就区分能力而言,还是传统的PFGE更有优势。

2.4　PCR检测耐药基因和毒力基因

根据KPX的全基因组测序结果,选择在50株临床分离株中检测耐药基因KPC-2、NDM-1、SHV-11、TEM-1、CTX-M、oqxB1、qnrS、sul2、sul1、int1,毒力基因iutA、iucABCD和rmpA2。这些基因的扩增结果如表2所示。50株中有49株检出KPC-2和SHV-11,38株检出TEM-1和CTX-M。同时携带3种A类β-内酰胺酶基因(SHV-11、TEM-1、CTX-M)的有30株。所有菌株均未检出NDM。66%的菌株检出Oqxb1,QnrS在菌株中的检出率为70%。Ⅰ类整合酶基因(Int1)检出率为42%,磺胺类耐药基因sul1和sul2

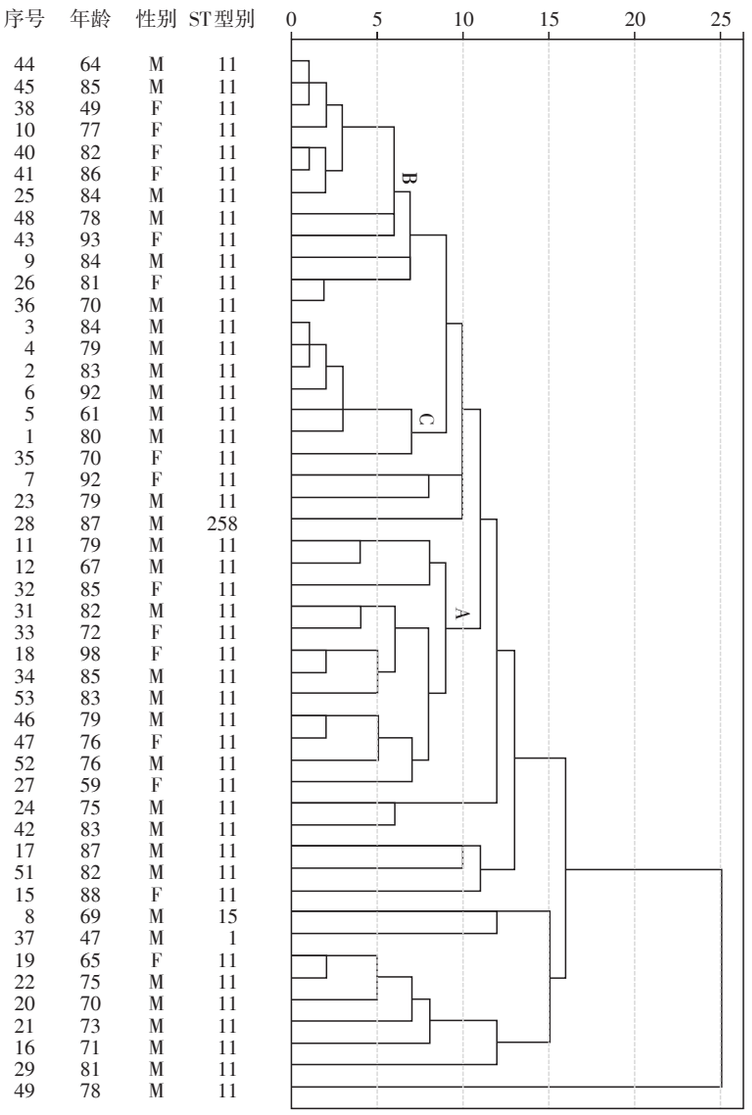

图5　MSLT及PFGE结果

Figure 5　Results of MSLT and PFGE

表2 碳青霉烯类耐药肺炎克雷伯菌中耐药基因和毒力基因分布

Table 2 D istribution of resistance and virulence genes in carbapenem resistant Klebsiella pneumoniae

序号	KPC2	SHV11	TEM1	CTX-M	NDM1	oqxB1	qnrS	sul2	sul1	int1	iutA	iucC	rmpA2
1	+	+	+	−	−	−	−	+	−	+	+	+	+
2	+	+	−	−	−	−	−	+	+	−	+	−	+
3	+	+	+	+	−	+	+	+	−	+	+	−	+
4	+	+	+	+	−	+	−	+	−	+	+	−	+
5	+	+	−	−	−	−	−	−	+	+	+	−	+
6	+	+	+	+	−	+	+	+	−	+	+	−	+
7	+	+	+	+	−	+	+	+	+	−	+	−	+
8	+	+	−	+	−	+	+	+	+	−	+	−	+
9	+	+	+	−	−	−	+	+	+	−	+	−	+
10	+	+	+	+	−	+	+	+	+	+	+	−	+
11	+	+	+	+	−	+	+	+	+	−	+	−	+
12	+	+	+	−	−	−	+	−	+	+	+	−	+
13	+	+	+	+	−	+	+	+	+	−	+	−	+
15	+	+	−	+	−	−	−	+	+	+	+	−	+
16	+	+	+	+	−	+	−	+	+	+	+	−	+
17	+	+	−	+	−	−	−	+	+	−	+	−	+
18	+	+	+	+	−	+	+	+	+	−	+	−	+
19	+	+	+	+	−	+	+	+	+	−	+	−	+
20	+	+	−	+	−	−	−	+	+	−	+	−	+
21	+	+	+	+	−	−	−	+	+	+	−	−	+
22	+	+	−	+	−	+	+	+	+	+	+	−	+
23	+	+	+	+	−	+	+	+	+	−	+	−	+
24	+	+	+	+	−	+	+	+	+	+	+	−	+
25	+	+	+	+	−	−	+	+	−	+	+	−	+
26	+	+	+	+	−	+	+	+	+	−	+	−	+
27	+	+	−	+	−	−	−	+	+	+	+	−	+
28	+	+	+	+	−	+	+	+	+	−	+	−	+
29	+	+	+	+	−	+	+	+	+	−	+	−	+
30	+	+	+	+	−	+	−	+	+	+	+	−	+
31	+	+	−	+	−	+	+	+	+	−	+	−	+
32	+	+	+	−	−	−	−	+	+	+	−	+	+
33	+	+	+	+	−	+	+	+	+	+	+	−	+
34	+	+	+	+	−	+	+	+	+	−	−	−	+
35	+	+	+	+	−	+	+	+	−	+	+	−	+
36	+	+	+	−	−	−	+	+	−	−	+	−	+
37	−	+	+	+	−	+	−	+	+	−	−	+	+
38	+	−	−	−	−	−	−	+	−	−	+	−	+
39	+	+	+	+	−	+	+	+	+	+	+	−	+
40	+	+	−	+	−	+	+	+	+	−	+	−	+
41	+	+	+	+	−	+	+	+	+	+	+	−	+
42	+	+	+	+	−	+	+	+	+	−	+	−	+
43	+	+	+	−	−	−	+	+	+	−	−	+	+
44	+	+	+	+	−	+	+	+	−	−	+	−	+
45	+	+	+	−	−	−	−	+	+	−	−	+	+
46	+	+	+	+	−	+	+	+	+	−	+	−	+
47	+	+	+	+	−	+	+	+	+	−	+	−	+
48	+	+	−	−	−	−	+	+	+	−	+	−	+
51	+	+	+	+	−	+	+	+	+	−	+	−	+
52	+	+	+	+	−	+	+	+	+	−	+	−	+
53	+	+	+	−	−	−	+	+	+	+	+	−	+

的检出率分别为82%和96%。41株sul1阳性的菌株中15株Int1也阳性。IutA和iucABCD的检出率分别为88%和10%，而rmpA2的检出率是100%。

3 讨　论

近年来肺炎克雷伯菌不仅在临床标本中的分离率越来越高，其对抗生素的耐药率也是越来越高，甚至有报道称出现了粘菌素耐药的菌株，因此研究肺炎克雷伯菌的耐药机制及其传播特点一直是微生物研究领域的热点[14]。肺炎克雷伯菌为环状闭合DNA，胞质内含有质粒，质粒是细菌染色体外的DNA分子，具有自主复制能力，携带多种编码细菌性状的遗传信息。质粒能在细菌间传播，赋予宿主菌某些生物学性状[15]。本研究选取的这株KPX胞质内共有4个质粒。根据测序结果显示染色体和质粒上的携带的耐药基因分别与细菌对氨基糖苷类、β内酰胺类、氟喹诺酮类、大环内酯类、磺胺类和氯霉素的耐药有关。这与细菌的药敏结果基本一致。磺胺类耐药基因sul1和sul2在细菌中的检出率很高，但是现实中磺胺类耐药率并没有很高，推测这种基因型与表型不一致的原因是磺胺类药物肾毒性大，现在临床应用的很少，从而降低了耐药率的发生。此外，耐药的产生还与基因表达的量有关，在抗生素选择压力不大的情况下，耐药基因表达水平不高，所以导致磺胺类的耐药表型与基因型存在一定偏差，至于体外敏感体内是否敏感还需进一步验证。与磺胺类药物存在同样情况的还有氨基糖苷类药物。Oqxb1这个基因编码多种药物外排泵，是喹诺酮类药物耐药的主要原因，和它同类的OqxAB基因在革兰阴性杆菌中很常见，尤其是在肠杆菌科细菌中[16]，介导交叉耐药或是降低细菌对多种药物的敏感性，包括氯霉素、环丙沙星、恩诺沙星、诺氟沙星和替加环素[17,18]。QnrS基因的突变同样也是介导细菌对喹诺酮类的耐药[19]。先前有报道称毒力基因iutA与肠道外大肠埃希菌的耐药相关，并且在产CTX-M型超广谱β内酰胺酶(ESBLs)的菌株里很常见。但在本实验中并未发现两者之间的相关性[20]。毒力基因rmpA2的检出率是100%，该基因的上调与侵入性感染有关，该实验中只检测了该基因的携带率并未检测其表达率，在今后的研究中需要进一步完善[21]。

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