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PCR/16sRNA 联合核苷酸测序法检测化脓性脑膜炎病原菌的临床价值 *

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摘要 目的：探讨 PCR/16sRNA 联合核苷酸测序法在化脓性脑膜炎病原菌检测中的临床诊断价值。**方法：**选择 2016 年 4 月至 2017 年 2 月上海儿童医学中心临床考虑中枢感染的 43 例化脓性脑膜炎患儿的脑脊液标本，所有患儿标本同时进行培养，并行 PCR/16sRNA 联合核苷酸测序法检测，记录检测结果，并统计检测方法的灵敏度和特异度，以脑脊液培养检测结果为金标准，对比脑脊液培养和 PCR/16sRNA 联合核苷酸测序法的灵敏度和特异度。**结果：** 脑脊液培养的灵敏度为 21.7%，特异度为 100.0%；PCR/16sRNA 联合核苷酸测序的灵敏度为 69.6%，特异度为 95.0%；两者的灵敏度比较差异具统计学意义（P<0.05），而两者特异性比较差异无统计学意义（P>0.05）。PCR/16sRNA 联合核苷酸测序可检出脑脊液培养阴性的病原体。**结论：**PCR/16sRNA 联合核苷酸测序具有较高的灵敏度，可检出脑脊液培养阴性的病原体，且受抗菌药物影响小，可为临床早期提供化脓性脑膜炎的病原学依据，降低致死率及致残率。

关键词：化脓性脑膜炎；病原学诊断；聚合酶链反应；16Srna

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Clinical Value of PCR/16sRNA Combined With Nucleotide Sequencing Method in Detecting Purulent Meningitis Pathogens*

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ABSTRACT Objective: To investigate the clinical value of PCR/16sRNA combined with nucleotide sequencing method in the detection of purulent meningitis pathogens. **Methods:** Cerebrospinal fluid (CSF) specimens of 43 children with purulent meningitis in Shanghai Children's Medical Center from April 2016 to February 2017 were selected, all specimens were cultured simultaneously, which were detected by PCR/16sRNA sequencing combined with nucleotide sequencing. The detection results were recorded, and the sensitivity and specificity of the detection method were statistically analyzed, and the sensitivity and specificity of CSF culture and PCR/16sRNA combined nucleotide sequencing were compared with CSF culture test results as gold standard. **Results:** The sensitivity of CSF culture was 21.7%, the specificity was 100.0%, the sensitivity of PCR/16sRNA was 69.6%, the specificity was 95.0%, and the difference of sensitivity between the two methods was statistically significant (P<0.05), but there was no significant difference between the two methods (P>0.05). PCR/16sRNA combined with nucleotide sequencing could detect cerebrospinal fluid culture negative pathogens. **Conclusion:** PCR/16sRNA combined with nucleotide sequencing has higher sensitivity, it can detect cerebrospinal fluid culture negative pathogens, and less affected by antibiotics.It can provide the early clinical pathogen diagnosis of purulent meningitis, reduce mortality and morbidity.

Key words: Purulent meningitis; Pathogen diagnosis; PCR; 16Srna

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前言

脑膜炎是由细菌、真菌等多种病原体而引起的以中枢神经系统损害为主的严重的感染性疾病，多起病急，临床表现形式多样，致死率与致残率都较高[1,2]。近年来由于相关疫苗的研发与应用，发病率已有所下降，但是全球范围内每年仍有 120 万左右的新发病例，其中 30~40 万人死亡，致残率达 20~50%[3,4]。受地域以及致病病原体影响，各地区的发病率不一，但发展中国家普遍高于发达国家，婴幼儿高于成人，如中国全人群化脑的发病率波动于 1.84~2.93/100,0000，而小于 5 岁儿童的发病率则高达 6.95~22.3/100,0000[5,6]。早期、有效、个体化的抗感染治疗对改善预后至关重要。现阶段临床脑膜炎的诊断仍有赖于脑脊液常规与生化检查、脑脊液培养及血培养[7,8]。其中脑脊液培养是脑膜炎诊断的金标准，但由于腰椎穿刺前即经验性应用

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抗生素治疗，部分特殊细菌对培养环境的要求高，使得脑脊液培养阳性率较低，且培养时间需大于 72 小时，这些都干扰了临床医生早期针对性的治疗，进而影响致死率与致残率的下降。因此临床迫切需求研发、开展新型的检测手段以弥补这些缺陷，从而提高病原菌的检出率[9-11]。聚合酶链反应（Polymerase chain reaction, PCR）具有灵敏度高、检测耗时短、不受检测样本中抗菌及抑菌物质的干扰等优点，近年来在全球范围内逐步被开展 [12-14]。本研究收集了上海儿童医学中心开展脑脊液 PCR/16sRNA 联合核苷酸测序检验项目以来，脑脊液常规培养与 PCR 的应用情况，以探讨 PCR 对脑膜炎病原菌诊断的临床应用价值。

1 材料和方法

1.1 一般资料

选择 2016 年 4 月至 2017 年 2 月间于上海儿童医学中心住院治疗、临床考虑中枢感染、且脑脊液标本同时完成常规培养与 PCR/16sRNA 联合核苷酸测序的患儿，共计 43 例。纳入标准：均符合 2003 年人卫版诸福棠儿科化脓性脑膜炎诊断标准[15]；临床具有呕吐、头疼、发热、抽搐、体温异常、精神改变等症状，同时还伴有脑脊液改变及脑膜刺激征；患儿家属对本研究知情，并签署知情同意书。排除标准：合并先天畸形患儿；临床资料不全患儿；合并梅毒、巨细胞病毒感染患儿。

1.2 实验方法

1.2.1 实验试剂与仪器 基因组 DNA 提取试剂盒购自美国 Promega 公司；PCR 试剂（TaqDNA 聚合酶、4× dNTPs、10× buffer 等）购自加拿大 MBI 公司；DNA 扩增仪为美国 Bio-Rad 公司产品；vitek 全自动细菌分析仪及 API 鉴定系统购自法国生物梅里埃公司。

1.2.2 引物的设计 通过 Internet 检索 GenBank 中常见细菌 16S rDNA 序列、真菌 26S rDNA 序列，在其保守区选择两对通用引物。其细菌通用引物序列为 5'-AGAGTTTGATCMTGGCTCAG-3' 及 5'-TACGGYTACCTTGTTACGACTT-3'。真菌通用引物序列为 5'-GCATATCAATAAGCGGAGGAAAAG-3' 及 5'-GGTCCGTGTTTCAAGACGG-3'。

1.2.3 病原菌 DNA 的提取 采集的脑脊液按上海 QIAGEN 公司生产的基因组 DNA 提取试剂盒（QIAGEN DNA Purification Kit）操作说明书进行操作。

1.2.4 目的基因的 PCR 扩增 将标本 DNA 各 2 μL 作为扩增的模板，加入 PCR 反应体系。PCR 反应条件为：94 度预变性 2 分钟；94 度变性 30 秒，62 度退火 30 秒，72 度延伸 30 秒，共 35 人循环，最后 72 度 10 分钟结束反应；选取电泳结果为阳性的 PCR 产物（有单一的目的条带），使用下游引物 3r 做单向核苷酸序列测定，并用特异引物进行确认试验。

1.2.5 扩增产物的纯化及测序 由上海英骏生物技术有限公司完成，序列分析用 BLAST 软件。

1.3 观察指标

43 例患儿均完成腰椎穿刺，留取脑脊液标本，同时送检脑脊液培养及 PCR/16sRNA 联合核苷酸测序检验，并记录检测结果。同时收集临床资料，如腰穿前是否应用抗生素、临床诊断及预后等。

1.4 统计学方法

应用 SPSS 17.0 统计学软件对数据进行统计学分析，计数资料用例数或百分率(%)表示，组间比较采用 Fisher 确切概率法，以双侧 $P<0.05$ 表示差异具有统计学意义。

2 结果

2.1 一般情况的描述性分析

43 例患儿中，22 例确诊为化脓性脑膜炎，1 例为真菌性脑膜炎，2 例为无菌性脑膜炎，余 18 例为病毒性脑炎或非中枢感染。以下为确诊脑膜炎患儿一般情况的描述性分析，详见表 1。

2.2 脑膜炎患儿血培养、脑脊液培养及脑脊液 PCR/16sRNA 阳性率的比较

23 例化脓性及真菌性脑膜炎患儿中，血培养的阳性率为 39.1%，脑脊液培养的阳性率为 21.7%，脑脊液 PCR/16sRNA 的阳性率为 56.5%，三者阳性率比较差异有统计学意义（$P<0.05$）；其中抗生素的应用可降低脑脊液培养的阳性率，但两者阳性率比较差异无统计学意义（$P>0.05$）。具体详见表 2。

2.3 脑脊液 PCR/16sRNA 检测病原体分析

通过脑脊液 PCR/16sRNA 进行检测发现，在新生儿中最常见的病原体为无乳链球菌（25.0%），而非新生儿中亦以链球菌为主，其中包括肺炎链球菌、草绿色链球菌（36.3%）。

2.4 脑脊液培养及 PCR/16sRNA 的特异度与敏感度分析

脑脊液培养的灵敏度为 21.7%，特异度为 100.0%，脑脊液 PCR/16sRNA 联合测序的灵敏度为 69.6%，特异度 95.0%，两者灵敏度比较差异有统计学意义（$P<0.05$），而特异度比较差异无统计学意义（$P>0.05$）。详见表 4。

3 讨论

在脑膜炎中，早期的病原学诊断与及时、有效的个体化治疗，对预后致关重要，即使是短时的延误都有可能增加死亡率及后遗症的发生率[16-18]。作为诊断金标准的脑脊液培养存在易受抗菌抑菌药物干扰、特殊细菌培养要求高等缺陷，检出阳性率低，且培养时间长（大于 72 小时），待结果回报，可能临床已丧失治疗的最佳时机[19-21]。如在本研究中，68%的患儿在完成腰椎穿刺前已经验性应用抗菌药物，进而导致脑脊液培养的阳性率下降（25.0% vs 20.0%）；而 PCR/16sRNA 联合核苷酸测序检出的阳性率不受抗菌药物的干扰（50.0% vs 60.0%）。此外脑脊液 PCR 检出阳性率（56.5%）约是脑脊液细菌培养阳性率（21.7%）的 2.6 倍，具有较高的灵敏度（69.6%）及特异度（95.0%），同时具有检测时间短（小于 24 小时）、准确度高的特点，使得该检测方法可第一时间为临床提供病原学依据，进行个体化治疗。

但值得注意的是，在本次研究中，PCR/16sRNA 联合测序的灵敏度为 69.6%，但 Brouwer MC[22]等人对 2025 例肺炎链球菌所致的化脓性脑膜炎患儿进行分析，显示灵敏度达 100.0%，来自西非的一项研究也显示通过 PCR 检测埃博拉病毒的灵敏度达 99.3%[23]。灵敏度的下降与细菌载量低、脑脊液处理不及时有关；但也可能由于本次研究仅针对细菌及真菌，并未对病毒及其他特殊病原体进行检测有关，导致检出率下降，影响灵敏度，在未来的研究中应进一步完善。

表 1 脑膜炎患儿一般情况的描述性分析(%)

Table 1 Descriptive analysis of the general situation in children with meningitis(%)

Characteristic(n=25)		Percentage(Proportion)
Age	Newbore(< 28 d)	48.0%(12/25)
	Non neonatal(> 28 d)	52.0%(13/25)
Sex	Male	72.0%(18/25)
	Female	28.0%(7/25)
Clinical manifestation	Fever	84.0%(21/25)
	Convulsion	8.0%(2/25)
	Vomit	24.0%(6/25)
	Psychiatric symptoms(Irritability、Spiritual worse et al)	28.0%(7/25)
	Positive signs of nervous system	12.0%(3/25)
Application of antibiotics before lumbar puncture	Yes	68.0%(17/25)
	No	32.0%(8/25)
Basic disease	No	64.0%(16/25)
	Blood system disease	4.0%(1/25)
	Trauma, Operation history (Traffic injury, Post-surgical of Hydrocephalus or NEC)	24.0%(6/25)
	Kawasaki disease	8.0%(2/25)
Clinical outcome	Recovery	68.0%(17/25)
	Sequela (subdural collection of fluid et al.)	24.0%(6/25)
	Give up/Death	8.0%(2/25)

表 2 脑膜炎患儿血培养、脑脊液培养及脑脊液 PCR/16sRNA 阳性率的比较(%)

Table 2 Comparison of blood culture, cerebrospinal fluid culture and cerebrospinal fluid PCR/16sRNA positive rate in children with meningitis(%)

Test method	Positive rate	Positive rate of antibiotic use in lumbar puncture	Positive rate of non antibiotic use in lumbar puncture
Blood culture	39.1%(9/23)*	-	-
CSF culture	21.7%(5/23)*	25.0%(2/8)	20.0%(3/15)
CSF PCR/16sRNA	56.5%(13/23)	50.0%(4/8)	60.0%(9/15)

Note: Compare with PCR/16sRNA, *P<0.05.

表 3 脑脊液 PCR/16sRNA 检测病原体分析(%)

Table 3 pathogen analysis of CSF PCR/16sRN detection(%)

Types of children	Pathogen	Percentage(Proportion)
Newborn	*Streptococcus agalactiae*	25.0%(3/12)
	Escherichia coli	16.7%(2/12)
	Enterococcus	8.3%(1/12)
Non neonatal	Streptococcus(*Streptococcus pneumoniae, Streptococcus viridans, Streptococcus agalactiae*)	36.4%(4/11)
	Baumanii	9.1%(1/11)
	Candida albicans	9.1%(1/11)
	Opportunistic bacteria(*Finegoldia magna, Campylobacter urealyticum, bacteroides fragilis*)	9.1%(1/11)

本次研究通过脑脊液 PCR/16sRNA 联合测序方法检出真菌(白色念珠菌)及厌氧菌(大芬戈尔德菌)的感染,而该菌通过常规的脑脊液培养条件难以生长,从而致培养假阴性。国外的一些报道也有类似的发现,如来自荷兰的病例报道发现 1 例由

表 4 脑脊液培养及 PCR/16sRNA 的敏感度与特异度分析(%)

Table 4 Analysis of sensitivity and specificity of cerebrospinal fluid culture and PCR/16sRNA (%)

Test method	Sensitivity(Positive detection rate)	Specificity(Negative detection rate)
CSF culture	21.7%(5/23)	100.0%(20/20)
PCR/16sRNA	69.6%(16/23)	95.0%(19/20)
x^2	10.602	1.026
P	0.001	0.311

溶血二氧化碳噬纤维菌(多通过狗咬伤传播)的化脓性脑膜炎患儿[24];而 1 位美国的 14 岁男孩病程长达 4 个月,常规检测手段未发现致病病原体, 最终通过脑脊液 PCR/16sRNA 联合测序,检出螺旋体,并挽回了生命[25]。这些罕见、机会致病菌,通过传统的手段难以检出,而新型的诊疗手段为有效的靶向治疗提供了可能[26,27]。

与传统脑脊液培养特异度达 100.0%相比,PCR/16sRNA 联合测序的方法, 有假阳性的可能, 本次研究显示特异度为 95.0%。该例患儿腰椎穿刺损伤明显,脑脊液标本中红细胞含量较高(1880× 10⁶/L),最终 PCR/16sRNA 联合测序结果与血培养相符,均为鲍曼不动杆菌。而 Almeida SM 等人的一项研究也有同样的发现,在肠道病毒所致的病毒性脑炎中,没有红细胞的脑脊液通过 PCR 的病毒检出率为 26.0%, 而有红细胞的脑脊液检出率仅为 9.2%(P=0.001)[28]。这主要是由于其中含有的亚铁血红素及血红蛋白, 可以竞争结合 DNA 聚合酶的活性位点,从而导致假阳性或假阴性[29,30]。

通过本次研究,我们发现 PCR/16sRNA 联合核苷酸测序的检测方法可检出脑脊液培养阴性的病原体, 具有灵敏度高、耗时短等优点,可以为临床提供早期的病原学诊据,对儿童化脓性脑膜炎的早期诊断有一定的应用价值,可作为脑脊液培养的补充检测手段。

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