



淋病奈瑟菌药敏试验刃天青微量稀释法的建立与评价

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【摘要】目的 建立一种基于刃天青显色的淋病奈瑟菌药敏试验方法并对其进行评价。**方法** 在肉汤微量稀释法的基础上,添加刃天青作为活细菌指示剂,采用参考株WHO G进行试验以优化加入刃天青后细菌的培养时间,肉眼观察颜色变化以读取结果。用琼脂稀释法(金标准)和刃天青微量稀释法对3株淋球菌参考株和32株分离株分别进行阿奇霉素、头孢曲松和大观霉素最低抑菌浓度(minimum inhibitory concentration, MIC)的测定,从基本一致性(essential agreement, EA)(反映对MIC值的一致性)、分类一致性(category agreement, CA)(反映对耐药、中介和敏感判定的一致性)、极重大误差(very major error, VME)(反映假敏感性)和重大误差(major error, ME)(反映假耐药性)等指标进行分析,评价刃天青微量稀释法用于淋球菌药敏试验的准确性。CA率和EA率 $\geq 90\%$, VME率和ME率 $\leq 3\%$ 被认为是可接受的标准。**结果** 加入刃天青后反应6 h读取的结果与琼脂稀释法一致,以此参数建立刃天青微量稀释法。刃天青微量稀释法测量阿奇霉素、头孢曲松和大观霉素MIC结果的EA率分别为97.1%、91.5%和94.3%, CA率分别为88.6%、94.3%和94.3%, VME率均为0%, ME率分别为11.4%、5.7%和5.7%。**结论** 本研究建立的刃天青微量稀释法用于测量抗生素对淋病奈瑟菌MIC时,与琼脂稀释法具有良好的一致性,敏感结果可信度极高,仅通过肉眼观察即可方便读取结果。但应慎重对待耐药结果,需要进行参数的优化。

【关键词】 淋病奈瑟菌 药敏试验 刃天青微量稀释法

Establishment and Evaluation of a Resazurin-Based Microdilution Assay for Microbial Sensitivity Test of *Neisseria gonorrhoeae* LI Menghuan¹, YANG Guiqin¹, WANG Youwei², YONG Gang², WANG Hongren³, BIE Mingjiang^{1,4△}, WANG Guoqing^{1△}. 1. West China School of Public Health and West China Fourth Hospital, Sichuan University, Chengdu 610041, China; 2. Clinical Laboratory Center, Sichuan Academy of Medical Sciences & Sichuan Provincial People's Hospital, Chengdu 610072, China; 3. Department of Pathogen Biology, West China School of Basic Medical Sciences & Forensic Medicine, Sichuan University, Chengdu 610041, China; 4. Editorial Board of Journal of Sichuan University (Medical Sciences), Chengdu 610041, China

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【Abstract】 Objective To establish and evaluate a microbial sensitivity test method for *Neisseria gonorrhoeae* based on resazurin coloration. **Methods** Based on the broth microdilution method, resazurin was added as a live bacteria indicator. WHO G, a WHO gonococcal reference strain, was used to optimize the incubation time for resazurin-stained bacteria and the color change was visually observed to obtain the results. Agar dilution method (the gold standard) and resazurin-based microdilution assay were used to determine the minimum inhibitory concentration (MIC) of azithromycin, ceftriaxone, and spectinomycin for 3 reference strains and 32 isolates of *Neisseria gonorrhoeae*. The results were analyzed based on essential agreement (EA), which reflected the consistency of the MIC values, category agreement (CA), which reflected the consistency in the determination of drug resistance, intermediary, and sensitivity, very major error (VME), which reflected false sensitivity, and major error (ME), which reflected pseudo drug resistance, to evaluate the accuracy of resazurin-based microdilution assay as a microbial sensitivity test of *Neisseria gonorrhoeae*. CA and EA rates $\geq 90\%$ and VME and ME rates $\leq 3\%$ were found to be the acceptable performance rates. **Results** The results obtained 6 hours after resazurin was added were consistent with those of the agar dilution method and the resazurin-based microdilution assay was established accordingly based on this parameter. The EA of resazurin-based microdilution assay for measuring the MIC results of azithromycin, ceftriaxone, and spectinomycin was 97.1%, 91.5%, and 94.3%, respectively, and the CA was 88.6%, 94.3%, and 94.3%, respectively. The VME was 0% for all three antibiotics, while the ME was 11.4%, 5.7%, and 5.7%, respectively. **Conclusion** The resazurin-based microdilution assay established in this study showed good agreement with agar dilution method for measuring the MIC of antibiotics against *Neisseria gonorrhoeae*. Moreover, the sensitivity results of this method were highly reliable and could be easily obtained through

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naked eye observation. Nonetheless, the results of drug resistance should be treated with caution and the optimization of parameters should be continued.

【Key words】 *Neisseria gonorrhoeae* Microbial sensitivity test Resazurin-based microdilution assay

淋病是由淋病奈瑟菌(*Neisseria gonorrhoeae*, 又称为淋球菌)感染引起的一种常见性病, 目前尚无有效疫苗, 主要依靠抗生素治疗, 但耐药现象极为严重^[1]。近些年来, 国际上多个国家和地区都报道了对一线治疗药物头孢曲松耐药的淋球菌^[2], 甚至包括多药耐药的淋球菌^[1], 对当前的淋病治疗方案造成了严重威胁。为控制耐药淋球菌的广泛传播, 快速、简单、准确的淋球菌耐药检测技术和方法尤为重要。目前用于淋球菌耐药检测的方法主要有琼脂稀释法、纸片扩散法(K-B法)、E-test法、肉汤微量稀释法(microbroth microdilution method)、核酸检测法(nucleic acid amplification test, NAAT)以及全基因组测序(whole genome sequencing, WGS)^[3-6]等。其中琼脂稀释法是测定抗菌药物对淋球菌最低抑菌浓度(minimal inhibitory concentration, MIC)的金标准, 也是目前在中国淋球菌耐药监测计划(China Gonococcal Resistance Surveillance Program, China-GRSP)中常规使用的方法^[7-8]。然而, 琼脂稀释法操作繁琐, 用时较长, 一般适合在参比实验室对大量菌株进行批量检测。纸片扩散法、NAAT和WGS对细菌的耐药性有比较准确的判定, 但难以进行定量检测^[3-4], 且NAAT和WGS难以发现新现(emerging)的耐药突变。E-test操作简单, 且可定量检测MIC, 在临床检测中具有一定的优势, 但价格昂贵, 且尚未获得中国食品药品监督管理局(CFDA)的批准^[8]。微量稀释法采用液体培养基在96孔板中进行培养, 通过肉眼观察孔内浊度或测量OD值来判定MIC值, 该方法相较琼脂稀释法更为简单、快速^[9-10], 也能得到比较准确的结果, 但若用肉眼观察来确定折点(breakpoint)时, 对操作者的经验要求较高。

刃天青(resazurin)是一种氧化还原染料, 对细菌无毒, 可被活细菌转化成粉红色的荧光染料试卤灵(resorufin), 因此被广泛应用于多种细菌的药敏试验中^[11-15]。本研究在微量稀释法的基础上, 添加刃天青作为活细菌指示剂, 通过肉眼观察颜色变化来确定折点, 建立一种简单、快速、准确的淋球菌药敏试验方法, 并以琼脂稀释法的MIC结果作为金标准进行对比, 对刃天青稀释法进行评价。

1 材料与方法

1.1 菌株

淋球菌参考菌株WHO G、WHO K和WHO P^[16]来自中国疾病预防控制中心性病控制中心。32株分离株来自

四川省人民医院临床医学检验中心。以上菌株均已采用琼脂稀释法进行了MIC测定^[17]。

1.2 主要试剂与仪器

大观霉素、阿奇霉素、头孢曲松均购自上海麦克林生化科技有限公司; 淋球菌液体培养基(GCBL)购自上海哈灵生物有限公司; GC琼脂培养基购自英国OXOID公司; 刃天青购自上海国药集团。紫外分光光度计购自美国Bio-Rad公司; 高速离心机购自德国Eppendorf公司; 超净工作台购自苏州净化厂; 二氧化碳恒温培养箱购自新加坡SANYO公司; 生物安全柜购自海尔公司; Milli-Q超纯水仪购自美国Millipore公司; 立式压力蒸汽灭菌器购自上海云泰仪器仪表有限公司; 96孔细菌培养板、6孔板、1.5 mL离心管等购自Corning公司。

1.3 琼脂稀释法测定抗生素MIC

琼脂稀释法是WHO推荐的测定抗菌药物对淋球菌MIC的金标准^[8]。将抗菌药物对倍稀释成一系列的浓度, 配制成含相应药物浓度的GC琼脂培养基。复苏冻存的细菌, 置于36℃、体积分数5%CO₂条件下培养16~18 h。传代2次后, 制成1.5×10⁸ CFU/mL的菌悬液, 然后用细菌点种仪将各株细菌的菌悬液点种于各药物浓度梯度的GC琼脂平板, 36℃、体积分数5%CO₂条件下培养24 h后观察结果, 以细菌不生长的最低药物浓度为该药物的MIC^[17]。

1.4 刃天青微量稀释法的建立与优化

本研究在WU等^[9]建立的微量稀释法的基础上, 加入刃天青作为活细菌指示剂, 通过对条件的简单优化, 建立起通过肉眼观察颜色变化的淋球菌药敏试验方法。首先在96孔板内制备对倍稀释的抗生素液, 每孔体积100 μL。然后将对数生长期的细菌(参考株WHO G)制成菌悬液, 调整浓度为5×10⁵ CFU/mL, 在96孔板中每孔加入100 μL。阴性对照孔(-)内不加菌液, 阳性对照孔(+)内加菌液不加抗生素, 每个浓度梯度设3个平行组。当观察到阳性对照孔出现浑浊后每孔加入50 μL无菌0.01%刃天青钠溶液, 继续培养。在培养后3 h、6 h、12 h和18 h各时间点观察颜色变化, 做好记录。以琼脂稀释法的MIC结果(阿奇霉素对WHO G的MIC=0.25 μg/mL)为参考, 用以确定刃天青加入后颜色变化的最佳时间点。

1.5 刃天青稀释法用于淋球菌药敏试验的评价

采用琼脂稀释法和刃天青微量稀释法, 分别对3株参

考株以及32株分离株进行阿奇霉素、头孢曲松和大观霉素三种抗生素的MIC测定,从基本一致性(essential agreement, EA)、分类一致性(category agreement, CA)、极重大误差(very major error, VME)和重大误差(major error, ME)等指标^[18]对刃天青微量稀释法进行评价。EA即待测方法与金标准相比MIC值不超过1个对倍稀释度的比例,CA为被评估的药敏方法与参考方法相比,判断结果为敏感、中介和耐药的一致性,VME和ME分别反映假敏感率和假耐药率。一般认为CA率、EA率 $\geq 90\%$,VME率和ME率 $\leq 3\%$ 被认为是可接受的标准^[8]。当CA率、EA率越高,则表明新方法测定的MIC结果与金标准的结果相同的概率越高,即新方法的敏感可信度越高。

2 结果

2.1 刃天青反应时间的确定

在96孔板中加入刃天青之后继续培养,在3 h、6 h、12 h和18 h各时间点观察颜色变化,得出的MIC值分别是0.0625 $\mu\text{g}/\text{mL}$ 、0.25 $\mu\text{g}/\text{mL}$ 、1 $\mu\text{g}/\text{mL}$ 和1 $\mu\text{g}/\text{mL}$ (如图1所示),因此加入刃天青后反应6 h的结果与琼脂稀释法一致,后续实验选用该条件对刃天青微量稀释法进行评价。将该方法用于32株阿奇霉素、头孢曲松和大观霉素MIC的测定,以刃天青反应6 h后颜色不变的最低药物浓度为该药物的MIC。以分离株SC18-25(经琼脂稀释法测定的准确结果)为例,采用刃天青稀释法测定阿奇霉素、头孢曲松和大观霉素MIC分别为0.5 $\mu\text{g}/\text{mL}$ 、0.5 $\mu\text{g}/\text{mL}$

和32 $\mu\text{g}/\text{mL}$ (如图2所示),而琼脂稀释法测定的结果分别为0.5 $\mu\text{g}/\text{mL}$ 、0.5 $\mu\text{g}/\text{mL}$ 和16 $\mu\text{g}/\text{mL}$,仅大观霉素的结果差了一个稀释度,其余均完全一致。

2.2 刃天青微量稀释法与琼脂稀释法之间的比较

以琼脂稀释法为金标准,评价刃天青稀释法用于淋球菌药敏试验的准确性。分析2种方法测定3种抗生素对淋球菌的MIC值,计算出刃天青稀释法测量阿奇霉素、头孢曲松和大观霉素MIC结果的EA率分别为97.1%(34/35)、91.5%(32/35)和94.3%(33/35)。见表1和表2。

3 讨论

淋球菌耐药已经成为了全人类面对的一个重大的公共卫生问题,快速检测细菌的耐药性是应对其耐药性传播的主要措施之一。世界卫生组织建议使用琼脂稀释法进行淋球菌药敏试验,但该方法操作繁琐,费时费力,仅适合批量检测;再加上抗生素平板的保存期限较短(不超过5 d)^[8],更加限制了该法在临床检测细菌耐药中的应用。数据显示,2018年China-GRSP采集的进行MIC检测的淋球菌临床分离株不足总数的1.8%(2 344/133 156)^[8]。因此需要改进当前淋球菌药敏试验的方法,以满足淋球菌耐药监测、临床检测等需求。

本研究在微量稀释法的基础上建立了刃天青微量稀释法,该方法可用肉眼观察颜色变化来确定细菌的存活状态,从而判断抗生素的MIC,提高了监测的敏感性,且不需要酶标仪即可进行结果读数,极大地简化了操作。

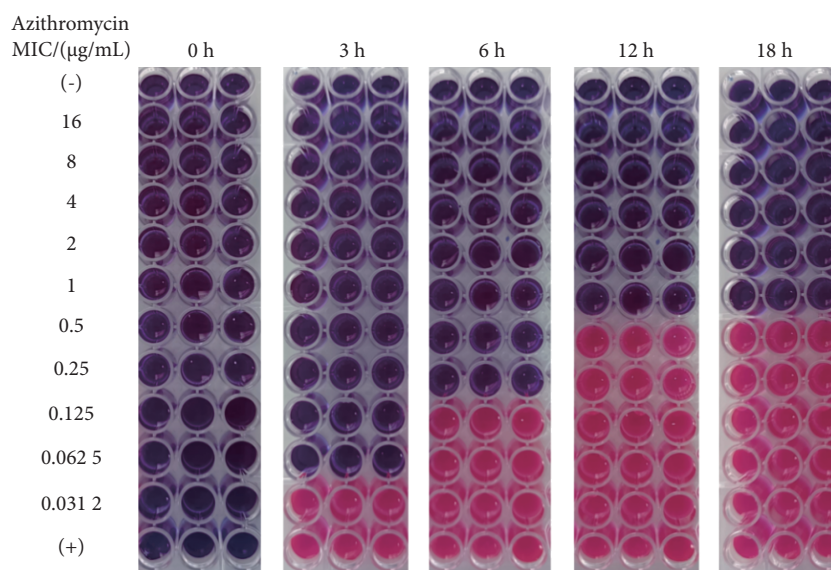


图1 刃天青反应不同时间的颜色变化(参考株WHO G)

Fig 1 Color changes of resazurin after different period of reaction time (reference strain WHO G)

(-): Negative control treated with bacterial solution; (+): positive control treated with bacterial solution, but not antibiotics; there are three parallel groups for each concentration gradient.

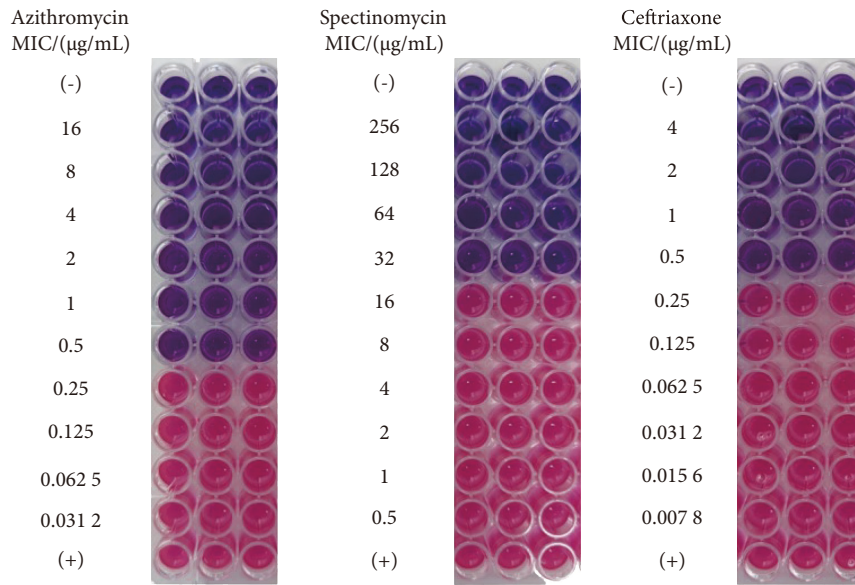


图 2 刃天青微量稀释法测定抗生素对分离株SC18-25的MIC结果

Fig 2 Resazurin microdilution method for determining MIC results of antibiotics against isolate SC18-25

(-): Negative control not treated with bacterial solution; (+): positive control treated with bacterial solution, but not antibiotics; there are three parallel groups for each concentration gradient.

表 1 刃天青稀释法和琼脂稀释法测定3种抗生素对淋球菌的MIC值的基本一致性 (EA)

Table 1 Essential agreement (EA) of MIC values of three antibiotics against *Neisseria gonorrhoeae* determined by resazurin-based microdilution method and agar dilution method

Difference in MIC	Azithromycin (n=35)		Ceftriaxone (n=35)		Spectinomycin (n=35)	
	Strain	Percentage	Strain	Percentage	Strain	Percentage
<-2	0	0.0%	0	0.0%	0	0.0%
-2	0	0.0%	0	0.0%	1	2.9%
-1	2	5.7%	3	8.6%	1	2.9%
0	24	68.6%	24	68.6%	18	51.4%
1	8	22.8%	5	14.3%	14	40.0%
2	0	0.0%	1	2.8%	2	5.7%
>2	1	2.9%	2	5.7%	0	0.0%
Total	34	97.1%	32	91.5%	33	94.3%

表 2 刃天青稀释法和琼脂稀释测定抗生素对淋球菌MIC结果的比较

Table 2 Comparison of *Neisseria gonorrhoeae* MIC findings measured with the resazurin-based microbroth dilution method versus agar dilution method

Index	Azithromycin (n=35)		Ceftriaxone (n=35)		Spectinomycin (n=35)	
	Strain	Percentage	Strain	Percentage	Strain	Percentage
EA	34	97.1%	32	91.5%	33	94.3%
CA	31	88.6%	33	94.3%	33	94.3%
VME	0	0.0%	0	0.0%	0	0.0%
ME	4	11.4%	2	5.7%	2	5.7%

刃天青是一种非特异性氧化还原指示剂,在一定数量活细菌存在时即可使其颜色发生变化,因此添加刃天青之后的不同时间点可能会得到不同的结果,本研究也证实了如此。通过对添加刃天青之后细菌培养时间的优化,我们确定了6 h为读取结果的最佳时间,而传统的琼脂稀释法需要培养24 h。因此,本研究建立的新方法相比传统的琼脂稀释法要更加快速,这对于临床检测细菌耐药性的时效性来说是十分重要的,为临床精准用药提供了一种快速选择。

除了快速,对于一种检测耐药性的方法来说,准确性更加重要。根据我国卫生行业标准^[18],本研究采用琼脂稀释法作为金标准,分析两种方法检测阿奇霉素、头孢曲松和大观霉素三种抗生素对3株参考株和32株分离株的MIC结果,对新方法进行了准确性评价。结果显示三种抗生素的EA率均在90%以上,证明本研究建立的新方法对于MIC的测定比较准确。但是,在对MIC \pm 1的结果分析中发现, MIC+1的比例(14.3%~40.0%)的比例远高于MIC-1的比例(2.9%~8.6%),说明新方法测定MIC的结果略微偏高。在CA率的结果中,只有阿奇霉素略低于90%(88.6%),头孢曲松和大观霉素均为94.3%,说明新方法对于淋球菌耐药、中介和敏感的判定是比较准确的。三种抗生素的VME率均为0%,即新方法测定的假敏感率为0%;而ME率则在5.7%~11.4%之间,说明新方法的有一定的假耐药率,这个结果也与EA率的结果相对应,即新方法检测的MIC略偏高。这意味着当使用该新方法检

测淋球菌MIC时,敏感结果可信度极高,但应慎重对待耐药结果,尤其是阿奇霉素的结果,可用琼脂稀释法进一步验证。同时,可对刃天青反应时间(3~6 h之间)进一步优化,以达到与琼脂稀释法更高的一致性。

综上所述,本研究建立了一种刃天青微量稀释法用于检测淋球菌的耐药性,测得的MIC与琼脂稀释法测量的结果具有良好的一致性,且更为简便、直观,显色6 h后就可以得出药敏结果。该方法在实际应用中也不需要酶标仪等检测仪器,仅通过肉眼观察即可方便读取结果,适合推广到基层医疗机构检测淋球菌的耐药性。另外,与琼脂稀释法适合批量检测相比,该方法既可批量检测,也可进行小量样本甚至单个样本的药敏试验,适用于临床实验室检测淋球菌耐药性,及时为临床医生提供用药参考。通过对比,本方法尽管存在一些缺陷,比如检测MIC略偏高,但总体准确性较好,为淋球菌耐药监测和耐药检测提供了一种新的方法选择。

* * *

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