·实验室研究·

汉坦病毒核蛋白的重组表达及其免疫印迹法在肾综合征出血热血清抗体检测中的应用

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【摘要】 目的　应用基因工程技术，在昆虫细胞中表达汉坦病毒（HV）S基因，表达产物作为诊断抗原，用于检测血清中抗HV特异性抗体IgG。**方法**　PCR扩增HV-Z10株N蛋白（NP）编码基因，基因工程方法构建NP编码基因昆虫表达系统rBAC-Z10S-TN。间接荧光法了解重组NP（rNP）表达及与特异性免疫反应情况，SDS-PAGE观察重组蛋白表达情况。建立免疫印迹法检测肾综合征出血热（HFRS）患者血清样品，并与常规间接免疫荧光法进行比较。**结果**　rBAC-Z10S-TN高效表达rNP，SDS-PAGE显示rNP的蛋白表达带，此抗原仅与HFRS患者血清起反应。经双盲试验，两法检测血清143份，HFRS阳性符合率为97.67%。**结论**　成功构建了HV-Z10株NP编码基因高效真核表达系统。所建立的免疫印迹法可作为HFRS简便、安全、敏感、特异的血清学诊断新方法。

【关键词】 汉坦病毒；N蛋白；重组表达；杆状病毒表达系统；免疫印迹法

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Recombinant expression of hantaan virus protein N with application of Western-blot in detecting anti-hantavirus antibody

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【Abstract】 Objective　S gene of hantavirus(HV) was expressed in insect cells by genetic engineering technology. The expression product of S gene was used as antigen to detect anti-HV specific antibody IgG in serum. **Methods**　Gene encoding NP of the strain HV-Z10 was amplified by PCR and then its eukaryotic expression system rBAC-Z10S-TN was constructed by using the routine genetic engineering method. SDS-PAGE was applied to measure the expression of rNP.Ion-exchange plus Ni-NTA-affinity chromatography was performed to purify the recombinant product. Indirect immuno-fluorescence assay (IFA) was used to determine the specific immune-reactivity of rNP. WB assay was established to detect the serum samples from 95 confirmed HFRS patients. Parameters related to the outcomes of detection were compared with the routine HV-IgG IFA method. **Results**　rBAC-Z10S-TN was able to express rNP with high efficiency. The purified rNP only showed a single protein fragment in the gel after SDS-PAGE. HV IgG could efficiently recognize rNP and hybridize with the recombinant protein. 97.67% of the serum samples from the HFRS patients were positive confirmed by WB. **Conclusions**　We successfully constructed a high efficient prokaryotic expression system of NP encoding gene from hantavirus strain HV-Z10. WB assay which was established in this study could be used as a new serological test for HFRS diagnosis, thanks to the simplicity, safety, sensitivity and specificity of this method.

【Key words】 Hantavirus; Nucleocapsid protein; Recombinant expression; Baculovirus expression system; Western-blot

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肾综合征出血热(HFRS)是由汉坦病毒(HV)感染引起的急性传染病[1-2]。HV属布尼亚病毒科汉坦病毒属[3-4],为负链RNA病毒,含大(L)、中(M)和小(S)3个基因片段。其中L和M基因片段分别编码多聚酶、糖蛋白(GP)GP1和GP2;S基因片段编码N蛋白(NP)[5-6]。NP能诱导机体产生HV特异性IgG抗体[7-8],因而是HFRS血清学诊断的必需抗原。国内外学者构建了不同表达系统表达NP,以作为HFRS的血清学诊断抗原[9-10]。本研究从我国用于生产流行性出血热灭活疫苗的HV-Z10毒株基因组克隆S基因编码区并构建了NP真核表达系统,以重组NP(rNP)为抗原,建立免疫印迹法,检测143份临床血清样本,并对其检测效果与HV为抗原的常规间接免疫荧光法进行比较。

材料与方法

1. 主要试剂:Taq DNA聚合酶、限制性内切酶、胶回收试剂盒购自生工生物工程(上海)股份有限公司,羊抗人及兔抗鼠IgG抗体购自美国Sigma公司,Bac-to-Bac试剂盒及转染试剂Lipofectin购自美国Invitrogen公司,含HV-Z10株S基因的质粒pUCm-Z10S及杆状病毒表达系统由本实验室保存。

2. 细胞及病毒:Vero-E6、昆虫细胞TN,为本实验室保存。

3. 血清样本:共143份血清样本,其中36份来自2013—2015年浙江省各医院就诊的HFRS阳性样本和47份发热非HFRS患者血清样本,60份对照血清样本来自健康体检者,重组抗原免疫荧光法(IFA)检测HV IgG抗体阴性。

4. 重组杆状病毒rBAC-Z10S筛选:按试剂盒说明书操作筛选出重组杆状病毒rBAC-Z10S。同样方法筛选不含外源基因的重组杆状病毒rBAC作为阴性对照。

5. rBAC-Z10S的表达:

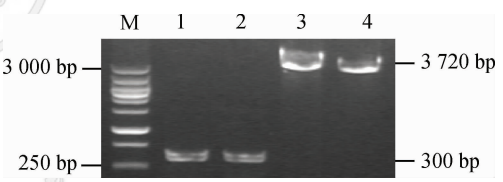
(1)IFA检测rNP的表达:按常规方法制备重组杆状病毒细胞抗原片。HFRS阳性血清按1∶2稀释成不同滴度,滴加抗原片,37 ℃孵育、洗涤,加FITC-羊抗人IgG(1∶32),37 ℃孵育、洗涤,吹干后,荧光显微镜下观察出特异性荧光者即为阳性。

(2)Western-bolt(WB)检测rNP的表达:将HV-rNP表达前、后的样品,SDS-PAGE电泳后,半干转移法转移至硝酸纤维素膜,封闭液(pH 7.5)室温作用1.5 h,置1∶2 000稀释的HRP标记兔抗HV IgG室温反应2 h,充分洗涤后加TMB避光显色10 min。

6. WB检测HFRS患者血清:收集感染rBAC-Z10S 3 d的TN细胞,SDS-PAGE后,半干转移至纤维素膜,制备成WB试纸条。按一定稀释度稀释的待检样品与WB试纸条于室温反应2 h后,洗膜后,加入1∶5 000稀释的碱性磷酸酶(AP)标记的羊抗人IgG抗体,室温反应2 h,洗膜,加显色液室温反应15 min。

结　果

1. 重组杆状病毒的鉴定:将2次筛选后的重组杆状病毒质粒,经PCR扩增,检测重组病毒质粒是否含有分子量在3 720 bp的HV-Z10 S基因表达单元(2 430 bp ＋1 290 bp ＝3 720 bp)。从图1可知,3、4泳道的质粒有约3 720 bp扩增条带,说明该质粒携有Z10 S基因的表达单元,1、2泳道约有300 bp的条带,为非重组病毒的质粒,最后选3道重组杆状病毒质粒进行转染昆虫细胞。



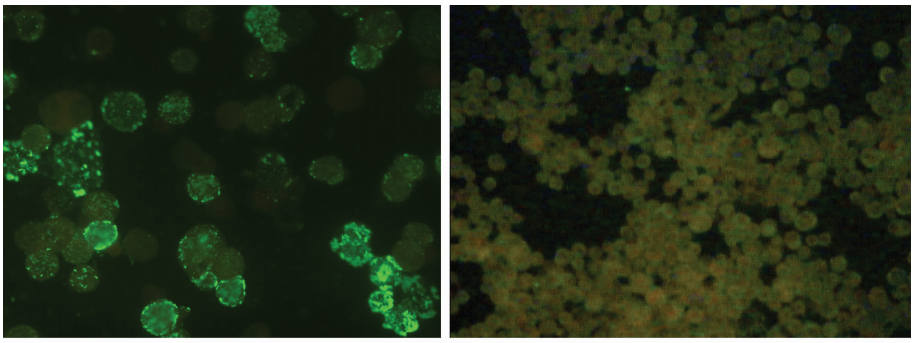
注:M为DNA标准分子量;1、2为非重组病毒质粒;3、4为重组病毒质粒

图1 PCR鉴定重组Z10 S基因的昆虫杆状病毒质粒

2. 间接免疫荧光检测重组蛋白HV-rNP的表达:将重组杆状病毒质粒转染昆虫细胞,5 d后收获上清,即为rBAC-Z10S,转种单层昆虫细胞,3 d后收获细胞滴加载玻片制备细胞抗原片,观察HV-rNP表达情况。抗原片与HFRS患者血清反应后,感染细胞的细胞质中出现不规则、点状特异性荧光(图2A);与正常人及非HFRS患者血清反应时,抗原片均未见上述特异性荧光(图2B)。

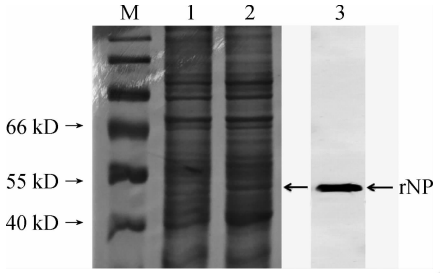
3. WB检测重组蛋白HV-rNP的表达:SDS-PAGE及WB结果显示,筛选的rBAC-Z10S重组杆状病毒能有效地表达HV-rNP(图3)。

4. WB待检血清稀释度的确定:选择HFRS弱阳性血清(间接免疫荧光滴度为1∶40),用WB检测,血清经1∶200、1∶500及1∶1 000稀释后均能检出阳性结果,最后确定1∶500作为WB检测HFRS患者



Ⓐ HFRS 阳性血清反应　　Ⓑ HFRS 阴性血清反应

图2　rBAC-Z10S与HFRS患者血清的间接免疫荧光结果(荧光显微镜×200)



注：M为蛋白标准分子量；1为重组杆状病毒rBAC；2为感染重组杆状病毒rBAC-Z10S的细胞超声液；3为rNP的Western-blot结果

图3　rNP表达及Western-blot结果

血清的稀释度。

5. 检测临床血清样本：143份血清样本，采用IFA检测HFRS阳性(IFA≥1∶20为阳性)36份，WB检测血清中的抗HV阳性抗体(WB≥1∶500为阳性)35份，灵敏度为97.2%；特异性为100.0%，与间接免疫荧光法比较，差异无统计学意义。

讨　论

本研究应用表达HV的rNP建立WB法，并对部分HFRS患者血清进行检测，与间接免疫荧光比较，检测143份血清两法符合率为97.67%，特异性为100.0%。表明WB与间接免疫荧光法对HFRS患者血清样品具有相同的检测效果。

与间接免疫荧法比较，WB法对不同血清稀释度的HFRS患者其检测值大大提高。荧光滴度为1∶40的弱阳性血清，WB可检测到1∶8 000。分析原因，可能是间接免疫荧法检测时，一抗二抗的反应时间均为30 min，抗原与抗体反应不够充分，而WB法检测时均为2 h，抗原抗体反应充分；其次由于WB反应在摇床中进行，抗原抗体能充分接触混合，也大大提高WB的检测水平。此外，间接免疫荧光试剂的制备需要用活的HV感染细胞，而重组杆状病毒对人无感染性，制备抗原也安全。因此，本研究建立的WB具有简便、安全、敏感、特异、可靠等优点，可作为HFRS患者血清学诊断方法。

利益冲突　无

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