

霍乱弧菌溶血素共调节蛋白(Hcp)的原核表达、纯化及多克隆抗体制备

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摘要: 目的 原核表达、纯化霍乱弧菌溶血素共调节蛋白(hemolysin coregulatory protein, Hcp), 并制备其多克隆抗体。方法 PCR扩增霍乱弧菌Hcp基因并克隆入pET28a载体中构建重组表达载体; 将重组载体pET28a-hcp转化*E. coli* BL21(DE3)中, 进行表达条件优化及表达形式鉴定。获取可溶性Hcp蛋白行Ni-NTA柱纯化, 纯化的Hcp蛋白免疫BALB/c小鼠以制备多克隆抗体, 并用间接酶联免疫吸附试验(ELISA)检测抗体效价, 以评估其免疫原性。再以Western blot法分析抗体对霍乱弧菌Hcp蛋白的特异性识别。**结果** 重组载体pET28a-hcp的酶切片段与预期相符, 测序结果与GenBank数据库中hcp基因序列一致, 成功构建pET28a-hcp重组质粒, 重组质粒经异丙基- β -D-硫代半乳糖苷(IPTG)诱导表达相对分子量为28kD的目的蛋白; 经Ni-NTA柱纯化后获得较纯的Hcp蛋白, 免疫小鼠可获得效价为1:512 000的抗Hcp多克隆抗体(anti-Hcp); Western blot鉴定结果显示anti-Hcp具有识别霍乱弧菌Hcp蛋白的特异性。**结论** 成功获得可溶形式表达的Hcp蛋白, 免疫小鼠后获得高效价的抗Hcp多克隆抗体, 为后续研究Hcp蛋白在非O1/非O139群霍乱弧菌T6SS致病过程中的作用奠定了基础。

关键词: 非O1/非O139群霍乱弧菌; 溶血素共调节蛋白; 原核表达; 多克隆抗体

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Prokaryotic Expression, Purification and Polyclonal Antibody Preparation of Hemolysin Co-Regulatory Protein (Hcp) from *Vibrio Cholerae*

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Abstract: **Objective** To explore prokaryotic expression, purification of hemolysin coregulatory protein (Hcp) of *Vibrio cholerae*, and preparation of its polyclonal antibodies. **Methods** PCR was used to amplify *Vibrio cholerae* Hcp gene and clone it into pET28a vector to construct recombinant expression vector. The recombinant vector pET28a-hcp was transformed into *E. coli* BL21 (DE3) for expression condition optimization and expression form identification. The soluble Hcp protein was purified by Ni-NTA column. The purified Hcp protein was used to immunize BALB/c mice to prepare polyclonal antibodies. The antibody titer was detected by indirect enzyme-linked immunosorbent assay (ELISA) to evaluate its immunogenicity. Western blot was used to analyze the specific recognition of antibodies to Hcp protein in *Vibrio cholerae*. **Results** The enzyme fragment digested by recombinant vector pET28a-hcp was consistent with the expected, the sequencing results were consistent with the Hcp gene sequence in the GenBank database, and the pET28a-hcp recombinant plasmid was successfully constructed. The recombinant plasmid was induced by isopropyl- β -D-thiogalactopyranoside (IPTG) to express the target protein with a relative molecular weight of 28 kD. The pure Hcp protein was obtained after purification by Ni-NTA column, and then Hcp polyclonal antibody (anti-Hcp) with a titer of 1:512 000 could be obtained from immunized mice. Western blot results showed that anti-Hcp had specificity in recognizing Hcp protein in *Vibrio cholerae*. **Conclusion** The soluble expression of Hcp protein is successfully obtained, and high-titer polyclonal antibodies against Hcp are obtained after immunization of mice, which may lay a foundation for subsequent studies on the role of Hcp protein in the pathogenesis of T6SS in non-O1/non-O139 *V. cholerae*.

Keywords: non-O1/non-O139 *Vibrio cholerae*; hemolysin coregulatory protein; prokaryotic expression; polyclonal antibody

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霍乱弧菌 (*Vibrio cholerae*) 是一种革兰氏阴性弧菌, 可通过食入受污染的食物或水传播给人类^[1]。根据其表面 O 抗原结构差异, 可分为 200 多种血清群。近年来, 非 O1/ 非 O139 血清群菌株主要引起胃肠炎和肠外侵袭性感染如菌血症、皮肤和组织感染、脑膜炎等^[2-4] 呈上升趋势。但是目前, 关于非 O1/ 非 O139 霍乱弧菌的致病机制, 人们还了解甚少。在非 O1/ 非 O139 菌株中发现了一些新的毒力因子如 VI 型分泌系统 (type VI secretion system, T6SS)、III 型分泌系统 (type III secretion system, T3SS)、溶血素 (hemolysin, HlyA)、热稳定肠毒素和血凝素等^[5-6]。

其中 T6SS 是一种多组分毒素递送装置, 一般由 13 个保守核心基因编码, 构成了其分泌系统的核心组件。在结构和功能上类似于 T4 噬菌体尾部刺突^[7]。T6SS 参与细菌的致病性, 并与应激反应和识别异己以及细菌耐药性的获得有关。其典型的结构特征由溶血素共调节蛋白 (hemolysin coregulatory protein, Hcp) 组成一个长的导管结构横跨整个细胞膜, 允许效应物质通过, 并可保护效应物质避免其被降解^[8]。编码霍乱弧菌 Hcp 的基因一般长度为 519 bp, 不同菌种的 Hcp 基因序列高度保守, 可表达分子量为 28 kD 的 Hcp 蛋白^[9]。Hcp 蛋白由 160 个氨基酸构成, 其二级结构高度保守, 三级结构类似, 只存在有微小的结构差异, 如在延伸环 (loop) 残基上存在遗传学差异^[10]。三级结构与噬菌体尾管蛋白 gp19/gp5 十分相似, 其外径约为 8 nm, 内径约为 4 nm 的六元环^[11]。由于有研究发现 Hcp 既可作为结构蛋白又可作为效应蛋白, 因此对其功能研究至关重要, 特别是在不同菌种内 Hcp 作用需要进一步的研究和认识^[12]。目前在非 O1/ 非 O139 群霍乱弧菌中 Hcp 的功能了解甚少, 本研究拟通过基因克隆技术构建 Hcp 蛋白表达载体, 表达和纯化 Hcp 蛋白, 并通过免疫小鼠制备抗 Hcp 多克隆抗体, 为后续对 Hcp 蛋白功能的探讨及阐述 T6SS 的作用机制至关重要。

1 材料与方法

1.1 研究对象 非 O1/ 非 O139 群霍乱弧菌 HN375 (中国典型培养物保藏中心 CCTCCAB2010-414), Hcp 高表达菌株 (Δ tagH) 为本实验室前期构建^[13], 大肠埃希菌 (*E. coli*) DH5a 和 BL21(DE3) 菌株及 pET28a 质粒为本实验室保存^[14]; BALB/c 小鼠, SPF 级, 6~8 周, 雌性, 购自遵义医科大学实验动物中心, 动物实验操作符合本校伦理委员会要求 (批准文号 KLL-2020-079)。

1.2 仪器与试剂 蛋白质 Maker, DL5000 DNA Maker, T4 DNA 连接酶, Primer Star 高保真 DNA 聚

合酶, 限制性内切酶和 DNA 纯化试剂盒 (TaKaRa, 日本); 质粒抽提试剂盒和细菌基因组 DNA 提取试剂盒 (TIANGEN, 北京); 辣根过氧化物酶 (HRP) 标记的山羊抗鼠抗体 (Proteintech, 武汉); Ni-NTA 亲和层析柱 (GE Healthcare, 美国); Omni-ECLTM 基础型化学发光检测试剂盒 (雅酶生物, 上海); MultisKan 型全波长酶标仪 (Thermo Scientific, 美国); FUSIONFX 凝胶成像分析仪 (Vilber Lourmat, 法国); 异丙基- β -D-硫代半乳糖苷 (IPTG)、卡那霉素 (Kanamycin)、LB 培养液等其他生化试剂购自生工生物工程 (上海) 股份有限公司^[14]。

1.3 方法

1.3.1 设计与合成引物: 根据 Hcp 基因序列, 设计了正向和反向引物分别为: F: 5'-CGCGGATCCATGCCAACTCCATGTTATATCTC-3' (下划线部分为 BamH I 酶切位点) 和 R: 5'-CCGCTCGAGTTACGCTTCGATTGGCTTACG-3' (下划线部分为 Xho I 酶切位点)。引物由生工生物工程 (上海) 股份有限公司合成。

1.3.2 PCR 扩增及回收 Hcp 基因: 提取霍乱弧菌 HN375 基因组为模板 PCR 扩增 Hcp, 扩增条件为: 98℃ 20s; 55℃ 15s; 72℃ 1min; 30 个循环; 72℃ 5 min。琼脂糖凝胶电泳鉴定 PCR 产物后纯化回收目的基因。

1.3.3 pET28a-hcp 原核表达载体的构建: 用 BamH I 和 Xho I 对 Hcp 基因及 pET28a 质粒双酶切, 对酶切产物鉴定、纯化回收和连接。再将连接产物转化入 *E. coli* DH5a, 通过菌液 PCR 和提取重组质粒双酶切筛选阳性克隆菌, 最后测序鉴定正确构建的 pET28a-hcp。

1.3.4 pET28a-hcp 重组质粒的诱导表达: pET28a-hcp 转化入 *E. coli* BL21(DE3), 菌落 PCR 鉴定出阳性克隆菌 [pET28a-hcp-*E. coli* BL21(DE3)]。将 pET28a-hcp-*E. coli* BL21(DE3) 接种于 LB 培养液 (含 Kanamycin, 50 μ g/ml), 37℃, 180 r/min 培养至 A_{600nm} 约为 0.6 时, 加入诱导剂 (IPTG) 终浓度为 0.2 mmol/L, 在 20℃, 100 r/min 条件下诱导 12 h, 离心收集菌体, 用超声缓冲液 (1 \times Binding Buffer) 重悬菌体和超声破碎菌体, SDS-PAGE 电泳检测上清和沉淀分析 Hcp 蛋白的表达形式。

1.3.5 纯化上清 Hcp 蛋白: 上清经 Ni-NTA 亲和层析柱纯化, 先后用含 10, 20, 30, 40, 300 和 500 mmol/L 咪唑的洗脱液洗脱, SDS-PAGE 电泳分析 Hcp 蛋白纯度。最佳洗脱浓度下的纯化蛋白超滤浓缩和去除 NaCl 及咪唑, 用二喹啉甲酸 (BCA) 法对浓缩蛋白定量和分装保存。

1.3.6 抗 Hcp 多克隆抗体的制备: 等体积混匀 Hcp 蛋白与 Alum 佐剂分别在 0, 14 和 28 天皮下免疫

BALB/c小鼠(30 μ g/只),同时设置免疫Alum佐剂作阴性对照组。首次免疫后35天采尾静脉血清分离血清检测抗体效价^[12]。间接酶联免疫吸附试验(ELISA)法测定抗体效价的结果判定标准为 A_{450nm} 处,以抗血清组/Alum佐剂对照组 ≥ 2.1 的最大稀释倍数判定为该抗体的滴度。

1.3.7 Western blot鉴定抗Hcp多克隆抗体的特异性:分别收集等量的霍乱弧菌HN375野生菌株(WT)和Hcp高表达菌株(Δ tagH)菌体,行SDS-PAGE电泳,转膜进行Western blot实验。抗Hcp多克隆抗体作为一抗,HRP标记羊抗鼠IgG作为二抗,FUSIONFX凝胶成像分析仪成像。

2 结果

2.1 PCR扩增Hcp基因及构建pET28a-hcp原核表达载体 见图1。PCR扩增Hcp产物经琼脂糖凝胶电泳检测显示与预期Hcp分子量519 bp一致的特异性条带。从菌液PCR阳性的克隆菌提取重组质粒双酶切,可见约519 bp的酶切片段,且重组质粒测序结果与Hcp基因序列相符,表明pET28a-hcp重组质粒构建成功。

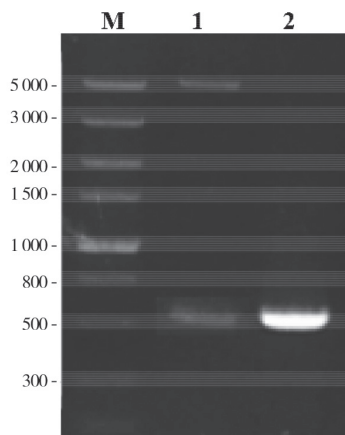


图1 PCR产物和重组质粒双酶切鉴定
M: DNA Marker DL5 000 bp; 1: pET28a-hcp 双酶切产物; 2: hcp 基因PCR扩增产物。

2.2 pET28a-hcp重组质粒中Hcp蛋白的诱导表达及纯化 见图2。IPTG诱导pET28a-hcp-*E. coli* BL21 (DE3),有分子量约为28 kD的Hcp蛋白表达,且为可溶性表达。Ni-NTA柱亲和层析纯化获较纯的Hcp蛋白。

2.3 抗Hcp多克隆抗体效价检测 见图3。Hcp蛋白经皮下末次免疫小鼠7天后取尾静脉血清检测抗体效价,结果显示阴性对照组没有检测到抗Hcp多克隆抗体,而实验组的抗血清效价达到1:512 000,表明Hcp蛋白有较强的免疫原性。

2.4 抗Hcp多克隆抗体特异性的鉴定 见图4。全菌WT和Hcp高表达菌株(Δ tagH)与一抗抗Hcp多克隆抗体行Western blot检测,在分子量约28 kD处

出现特异性反应条带。表明抗Hcp多克隆抗体能特异性识别霍乱弧菌Hcp蛋白。

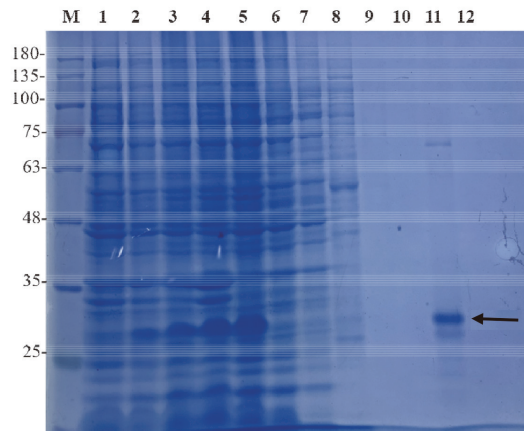


图2 pET28a-hcp在*E. coli* BL21(DE3)中诱导表达和Hcp蛋白纯化的SDS-PAGE分析
M: 180 kD蛋白质Marker; 1: 未诱导全菌; 2: 诱导全菌; 3: 诱导菌裂解液; 4: 诱导菌裂解沉淀; 5: 诱导菌裂解上清; 6: 穿液; 7~12: 分别为含10, 20, 30, 40, 300, 500 mmol/L咪唑的1×Washing Buffer经Ni-NTA柱后的洗涤液^[14]。

图2 pET28a-hcp在*E. coli* BL21(DE3)中诱导表达和Hcp蛋白纯化的SDS-PAGE分析

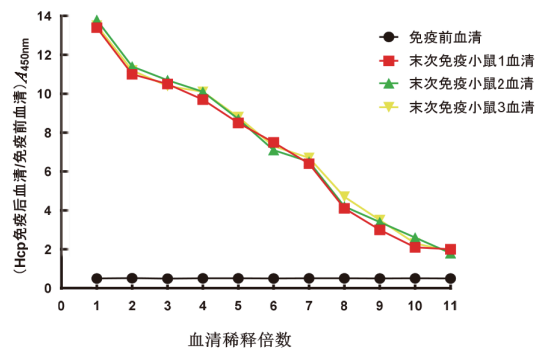


图3 Hcp蛋白免疫前后小鼠血清抗体效价分析

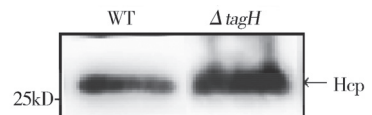


图4 Western blot分析抗Hcp多克隆抗体的特异性
3 讨论

溶血素共调节蛋白(Hcp)是一种在不同细菌中具有多样功能的分泌蛋白,可影响细菌的黏附能力、运动能力和生物膜形成,以及参与细菌间竞争的能力等^[15-16]。Hcp的分泌在同一菌种的不同菌株间也可能存在差异,有的菌株能分泌Hcp,而有的则不能^[17]。研究发现Hcp可作为T6SS的组分并受到T6SS的调控^[18]。近年来,许多研究者开始研究T6SS的Hcp蛋白及效应蛋白,由于Hcp既可作为结构蛋白又可作为效应蛋白,因此对其功能研究至关重要^[19]。而目前细菌T6SS引起致病的机制与Hcp蛋白发挥功能的分子机制仍然未全知,特别是在非O1/非O139群霍乱弧菌中。为了深入研究非

O1/非O139群霍乱弧菌中Hcp的功能,本研究以Hcp蛋白为研究对象,建立一种成本低廉、操作方便和表达效率高的Hcp蛋白表达系统,以表达可溶性的Hcp蛋白和制备抗Hcp多克隆抗体。表达外源蛋白的系统有大肠埃希菌表达系统、昆虫杆状病毒表达系统和毕赤酵母表达系统等^[20],其中大肠埃希菌原核表达系统是本研究首选的表达系统,因为大肠埃希菌容易培养、繁殖速度快、表达量高和成本低廉。目前广泛应用的原核表达载体有pET系列、pMAL系列、pGEX系列和pRSF系列等^[21],选择合适的表达载体对于目的基因在大肠埃希菌中的成功表达至关重要。由于pET表达载体亲和层析标签小,具有表达效率高,表达产物易被纯化,且在纯化后不需要对标签进行切除的优点,故本实验选用pET28a作为原核表达载体^[22]。

本研究通过基因工程技术以pET28a为载体成功构建Hcp原核表达系统,能够方便、快速和大量表达可溶性的Hcp蛋白。该蛋白能刺激小鼠产生较强的免疫应答和具有较好的免疫原性,免疫小鼠获得高效价的多克隆抗体。且Western blot结果显示抗Hcp多克隆抗体具有识别霍乱弧菌Hcp蛋白的特异性。

综上所述,本研究成功构建一种高效、方便的Hcp原核表达系统表达可溶性Hcp蛋白和制备能特异性识别霍乱弧菌Hcp蛋白的抗Hcp多克隆抗体,为后续非O1/非O139群霍乱弧菌中Hcp在T6SS中的作用研究奠定实验基础。

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