文章编号:1000 - 0615(2004)02 - 0183 - 06

斜带石斑神经坏死病毒外壳蛋白基因克隆与序列分析

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摘要:从患病毒性神经坏死病的斜带石斑鱼(*Epinephelus coioids*)的头部提取总 RNA,根据已发表的神经坏死病毒外壳蛋白基因设计引物进行 RT-PCR 扩增,得到预期大小的基因片段。将此基因片段转入 pET 载体进行序列测定和分析,结果表明:编码斜带石斑神经坏死病毒(Orange-spotted grouper nervous necrosis virus,OGNNV)外壳蛋白基因的阅读框核苷酸数为 1017bp,编码 338 个氨基酸;基因的核苷酸序列与野田村病毒科(Nodaviridae)的几种病毒的外壳蛋白基因序列比较结果显示,该病毒与β野田村病毒属(*Betanodavirus*)中的赤点石斑神经坏死病毒(red-spotted grouper nervous necrosis virus,RGNNV)的同源性最高(99 %),说明该病毒株是 RGNNV 血清型的成员。

关键词:斜带石斑;神经坏死病毒;外壳蛋白基因;野田村病毒科;β野田村病毒属
中图分类号:S917 **文献标识码**:A

Cloning and sequence analysis of the coat protein gene of nervous necrosis virus in Epinephelus coioides

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Abstract: The nodaviridae, which include two genera: alphanodavirus and betanodavirus, are a family of small, isometric and non-enveloped RNA viruses. Alphanodaviruses primarily infect insects and betanodaviruses infect fish. Their genomes, which are among the smallest known for animal viruses, consist of two co-encapsidated positive-sense RNA segments: RNA1 encodes the RNA-dependent RNA polymerase (RdRp) which replicates the viral genomes, while RNA2 encodes a precursor to the coat protein. Based on the coat protein sequence, betanodaviruses are categorized into four different genotypes: TPNNV (tiger puffer nervous necrosis virus), SJNNV (striped jack nervous necrosis virus), BFNNV (barfin flounder nervous necrosis virus), RGNNV (red-spotted grouper nervous necrosis virus). Orange-spotted grouper (*Epinephelus coioides*) nervous necrosis virus (OGNNV) is the causative agent of viral nervous necrosis (VNN) in larval and juvenal orange-spotted grouper fish. In this study, total RNA was extracted from the head of orange-spotted grouper infected with VNN. A pair of primers were designed according to the homogenus gene sequences, which have been submitted to GeneBank,

收稿日期:2003-03-17
资助项目:国家 863 项目资助(2001AA601010)
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from other fish nervous necrosis virus (NNV). The forward (sense) primer contained Sac Ⅰsite and the reverse (antisense) primer contained Hind Ⅲsites. One target fragment was amplified by means of reverse-transcriptase polymerase chain reaction (RT-PCR). After digestion with Sac Ⅰ and Hand Ⅲ, the RT-PCR products were ligated into the pET32 plasmid vector and transformed into *E. coli* BL21. The RT-PCR product and recombinant plasmid were sequenced, respectively. The coat protein gene of OGNNV was 1017 bases, encoded a protein of 338 amino acid with molecular mass of 37059.88 da, and with isoelectric point (IP) of 8.87. The sequence of the coat protein gene from OGNNV was submitted to GeneBank and the accession number was AF534998. The nucleotide sequence of the coat protein gene from OGNNV was aligned with four betanodaviruses: SJNNV, TPNNV, RGNNV, BFNNV and one alphanodavirus: BBV (Black beetle virus). Analyses indicated that the nucleotide length of the coat protein gene from OGNNV was six bases shorter than SJNNV and TPNNV, and that the length of encoded protein was 2 aa shorter than SJNNV and TPNNV, and that the length of nucleotide sequence and encoded protein was the same as RGNNV and BFNNV. The sequence similarities between the coat protein gene from OGNNV and four fish nodaviruses (SJNNV, TPNNV, RGNNV, BFNNV) was 76 %, 76 %, 81.4 % and 99 %, respectively. The coat protein gene from OGNNV had the highest identity of 99 % with RGNNV at nucleotide level, and had low identity of 22 % with BBV at nucleotide level. Phylogenetic comparisons of OGNNV were performed against five nodaviruses. Analyses showed that the coat protein sequence of OGNNV was closely related to four fish nodaviruses, and was most closely related to RGNNV, but was not related to BBV. Neutralization epitope analysis revealed that OGNNV shared the same PDG, which was completely preserved in RGNNV genotype, at aa 254 - 256 with RGNNV. These results suggested that OGNNV belonged to RGNNV genotype, but was different from insect nodaviruses.

Key words: *Epinephelus coioides*; nervous necrosis virus; coat protein gene; nodaviridae; *Betanodavirus*

海水鱼病毒性神经坏死病(viral nervous necrosis, VNN)或称视网膜病、空泡性脑病,是上世纪八十年代后期以来海水鱼育苗生产发生的一种死亡率极高的疾病[1],病毒可由亲鱼经性腺感染给仔鱼[2],幼鱼和稚鱼易发病,死亡率达 90 % 以上,甚至高达 100 %,成鱼也可患此病[3,4]。该病毒分布广泛,目前除非洲外,其他各洲都有报道。鳗鲡目、鲈形目、鲽形目、鲀形目、鳕形目中的十几个科的海水鱼类易受该病毒感染,已报道的受害鱼达 40 余种[3-7]。

野田村病毒科(Nodaviridae)包括α野田村病毒属(*Alphanodavirus*)和β野田村病毒属(*Betanodavirus*)两个属[8]。α野田村病毒是引起昆虫致病的病原体,引起海水鱼病毒性神经坏死病的病毒是β野田村病毒[9-10]。β野田村病毒粒子呈二十面体,无囊膜,病毒颗粒大小因感染鱼种不同有差异,通常在 25～30nm 之间。病毒基因组由两条单链 RNA 组成(RNA1 和 RNA2),其中 RNA1 编码非结构蛋白 —依赖 RNA 的 RNA 聚合酶;RNA2 含有一个开放阅读框,编码病毒外壳蛋白[11]。对 25 种 *Betanodavirus* 病毒外壳蛋白基因核苷酸序列的可变区进行同源性分析,Nishizawa 等[12]将其分为 4 种血清型,即 TPNNV (tiger puffer nervous necrosis virus)、SJNNV (striped jack nervous necrosis virus)、BFNNV (barfin flounder nervous necrosis virus) 和 RGNNV (red-spotted grouper nervous necrosis virus)。

斜带石斑鱼是重要的海水养殖品种,近年来由于受 NNV 感染,使育苗生产造成极大损失。本研究根据已发表的 VNN 的 RNA2 同源序列,设计了 1 对特异引物,从患病斜带石斑鱼扩增得到编码斜带石斑神经坏死病毒(Orange-spotted grouper NNV, OGNNV)的外壳蛋白基因,并对其序列进行详细分析。

1 材料与方法

1.1 材料

病毒来源 感染 OGNNV 的濒死斜带石斑鱼苗(孵出后 25～38d)于发病高峰时期取自广东省某水产养殖中心,表现出典型的神经坏死病毒病症状,经组织病理和电镜检测在其脑和视网膜发现大量受感染的阳性细胞和 OGNNV 粒子,PCR

检测为阳性,将之用液氮冷冻后带回实验室,-70 ℃保存。

宿主菌和质粒载体　大肠杆菌 BL21 和质粒 pET32 由第一军医大学彭红娟博士惠赠。

酶类和试剂　M-MLV 反转录酶购自 Gibco BRL 公司,pfu 酶购自上海博采生物有限公司,限制性内切酶购自 New England Biotech 公司,T4 连接酶购自华美生物工程公司,LB 培养基购自 Oxoid Ltd.,其它试剂均为国产分析纯。

引物　由上海基康生物技术公司合成。

1.2　方法

总 RNA 的提取　取被 OGNNV 感染的斜带石斑鱼苗的脑和眼,称重,剪碎,加适量的 Trizol B 匀浆,按 Trizol B 说明书提取总 RNA,-70 ℃保存备用。

目的片段的克隆　cDNA 的合成参照文献[13]等方法进行。回收的 cDNA 和空载体 pET32 用 *Sac* Ⅰ和 *Hind* Ⅲ分别进行双酶切,用 T4 连接酶连接,酶切和 PCR 筛选重组质粒。

测序　目的片段的序列测定,将 RT-PCR 产物和重组质粒送上海基康生物技术公司和上海博亚生物技术公司。

2　结果与分析

2.1　RT-PCR 扩增

根据 GenBank 数据库公布的 Dragon nervous necrosis virus 的 RNA2 核苷酸序列,设计 1 对特异性引物:

上游引物 F1:

5'-TCGAGCTCATGGTACGCAAAGGT-3'

下游引物 R1:

5'-GCCCAAGCTTTAGTTTTCCGAGTC-3'

其中,F1 和 R1 的 5'端的 TC 和 GCCC 均为加入的保护性碱基,并分别加入了 *Sac* Ⅰ和 *Hind* Ⅲ酶切识别序列。F1 中包含外壳蛋白基因的起始密码子 ATG,R1 中包含该基因的终止密码子 TAA。

RT-PCR 扩增结果如图 1 所示,在约 1kb 处有亮带出现,表明已得到与预期大小相符的片段。

2.2　重组质粒的酶切鉴定

如图 2 所示,将重组质粒经 *Sac* Ⅰ和 *Hind* Ⅲ双酶切后,得到大小约为 6kb 和 1kb 的两条片段,与预期的结果相符,表明此质粒是含插入片段的阳性重组子。

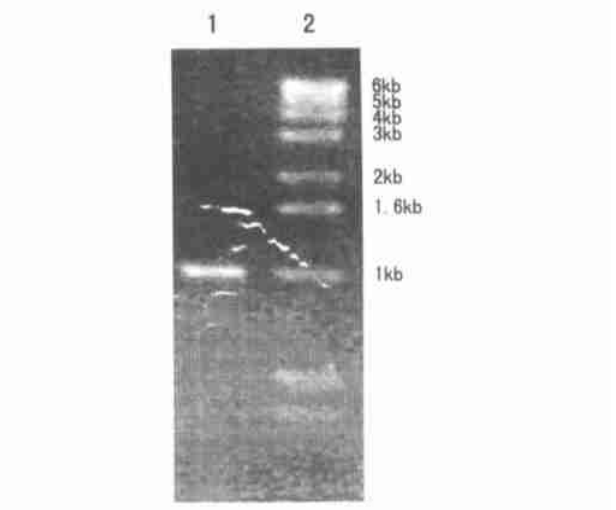

图 1　斜带石斑鱼神经坏死病毒外壳蛋白基因的 RT-PCR 扩增结果

Fig. 1　RT-PCR amplification of the coat protein of OGNNV

1. RT-PCR 产物;2. 1kb DNA 分子量标准

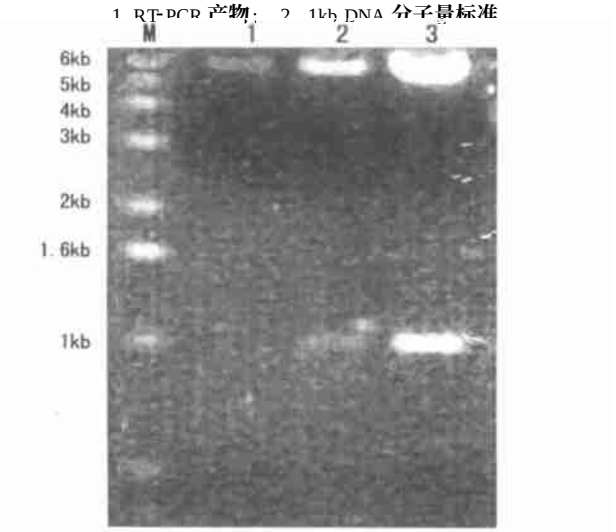

图 2　重组质粒的 *Sac* Ⅰ/ *Hind* Ⅲ酶切鉴定结果

Fig. 2　Characterization of the recombinant plasmids by *Sac* Ⅰ/ *Hind* Ⅲ digestion

1. 空载体 pET;2. 经 *Sac* Ⅰ/ *Hind* Ⅲ双酶切的重组质粒;M. 1kb DNA 分子量标准

2.3　测序

RT-PCR 产物与重组质粒的测序结果显示 1 段全长为 1017nt 的核苷酸序列。使用 DNATOOLS 软件对该序列进行分析,发现其开放阅读框(ORF)为 1017nt,编码 1 个由 338 个氨基酸组成,分子量为 37059.88da 的蛋白质,等电点(IP)为 8.87 (图 3)。序列已提交到 GeneBank 数据库,登录号为:AF534998。

2.4 同源性比较

利用 DNAStar 软件包中的 MegAlign 程序,将 OGNNV 外壳蛋白基因与 Alphanodavirus 中的 Black beetle virus (BBV),以及 *Betanodavirus* 的 4 种血清型的 NNV 外壳蛋白基因核苷酸序列进行同源性比较,即: SJNNV、TPNNV、BFNNV、RGNNV。结果显示如表 1,OGNNV 与 RGNNV 的同源性最高,达 99.0 %;与 SJNNV 和 TPNNV 的同源性为 76.0 %;与 BFNNV 的同源性为 81.4 %;而与 BBV 的同源性很低,只有 22.0 %

表 1 6 种 NNV 外壳蛋白基因核苷酸序列的同源性比较

Tab.1 Nucleotide similarities of the coat protein genes among six NNV

	OGNNV	BFNNV	RGNNV	SJNNV	TPNNV	BBV
OGNNV		81.4	99.0	76.0	76.0	22.0
BFNNV			81.2	73.7	76.5	20.4
RGNNV				75.3	76.0	21.3
SJNNV					77.6	19.9
TPNNV						21.1
BBV					9	

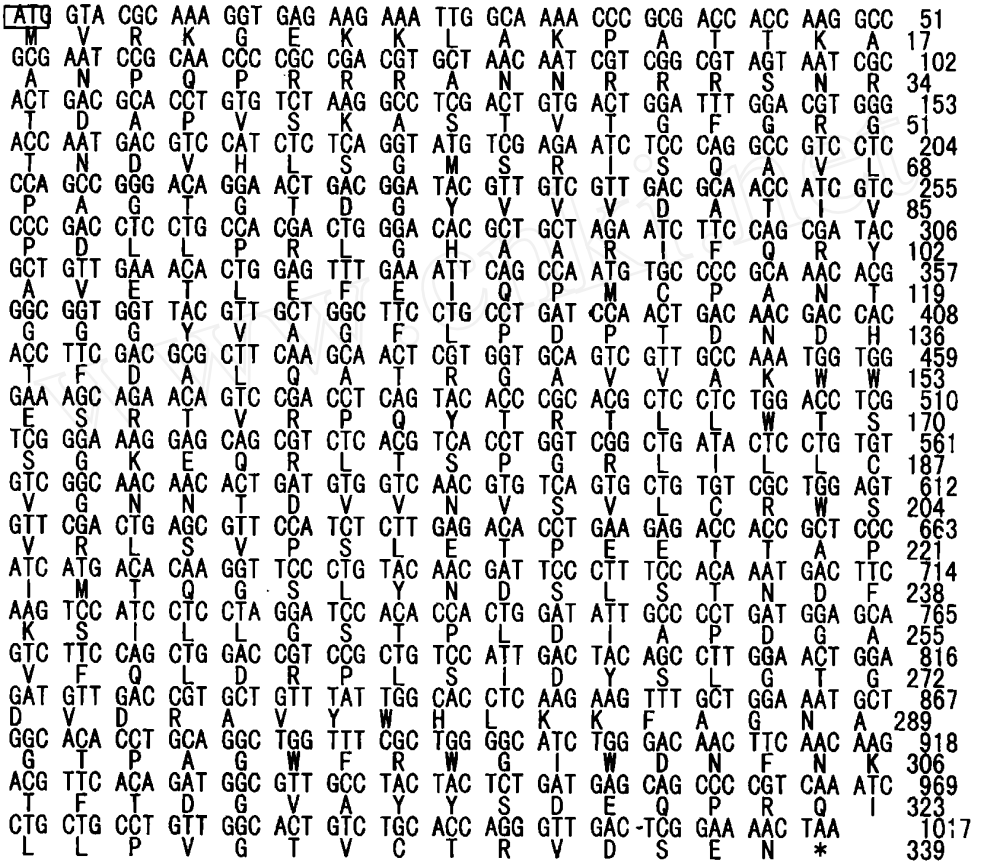

图 3 OGNNV 外壳蛋白基因核苷酸序列及其推导的氨基酸序列

Fig.3 Nucleotide sequence and deduced amino acid sequence of the coat protein gene from OGNNV

2.5 系统发育

利用 MegAlign 程序,根据 6 种 NNV 外壳蛋白基因编码的核苷酸序列构建的系统进化树如图 4 所示,可见 OGNNV 与 *Betanodavirus* 4 种血清型 NNV 的亲缘关系都较近,其中与 RGNNV 的亲缘关系最近;而与 BBV 的亲缘关系不近。

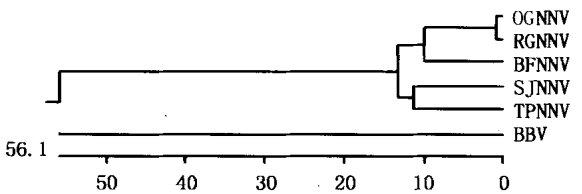

图 4 根据 6 种 NNV 外壳蛋白基因核苷酸序列建立的分子系统进化树

Fig.4 Molecular phylogenetic tree based on the nucleotide sequences of six isolates of fish nodaviruses

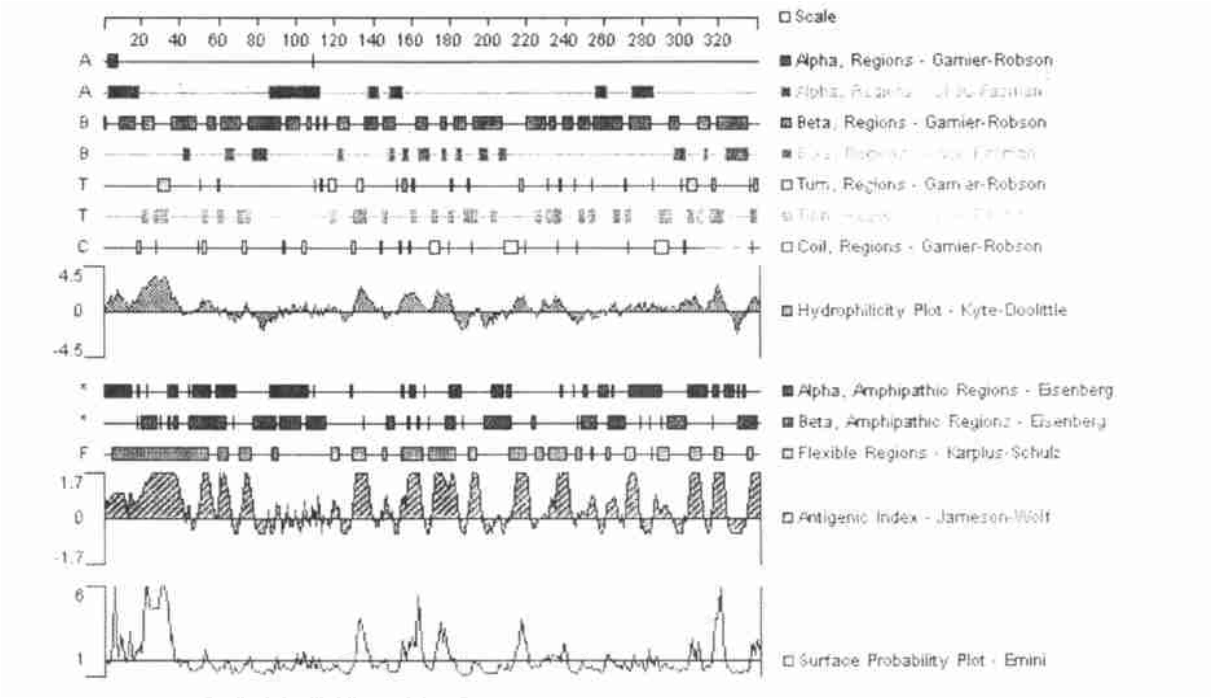

图 5　OGNNV 外壳蛋白基因编码的蛋白质的二级结构、抗原性及其疏水性/亲水性预测

Fig. 5　Secondary structure, antigenicity predictions and hydropathy plot of the coat protein gene of OGNNV

3　讨论

根据 NNV 外壳蛋白基因核苷酸序列比较,OGNNV 与 BBV 的同源性很低;分子系统进化分析显示,OGNNV 与 BBV 的亲缘关系也不近,由此可见,OGNNV 不属于 Alphanodavirus。

Betanodavirus 的 4 种血清型的病毒中,SJNNV 型和 TPNNV 型的外壳蛋白基因由 1023nt 核苷酸组成,编码 1 个由 340 个氨基酸组成的蛋白;而 RGNNV 型和 BFNNV 型的外壳蛋白基因核苷酸为 1017nt,在核苷酸序列的第 713～718 位置缺失了 6 个碱基,编码 1 个由 338 个氨基酸组成的蛋白[12,14,15]。本研究克隆的 OGNNV 外壳蛋白基因核苷酸序列长 1017nt,编码 1 个由 338 个氨基酸组成的蛋白;根据核苷酸序列比较和分子进化系统发育分析,OGNNV 与 RGNNV 亲缘关系最近。Nishizawa 等[16]分析 SJNNV 外壳蛋白基因上抗原中和表位的分布结果认为,其抗原中和表位很可能位于氨基酸序列 C 末端的可变区内,其 254～256 位的氨基酸残基最有可能是 1 个主要的抗原中和表位。SJNNV、TPNNV、BFNNV 和 RGNNV 的 254～256 位氨基酸残基分别是 PAN、PPG、PEG 和 PDG,并且这 3 个氨基酸残基在各种血清型中是绝对保守的。经分析 OGNNV 氨基酸序列发现在 254～256 位的氨基酸残基为 PDG,与 RGNNV 的完全相同。综上述,OGNNV 应属于 RGNNV 血清型的成员。Nishizawa 等[14]比较分析了 Nodaviridae 的几种病毒的外壳蛋白氨基酸序列特征,发现在靠近 N 端的前 50 个氨基酸残基中含有丰富的碱性氨基酸;我们分析 OGNNV 外壳蛋白基因推导的氨基酸序列,发现在 N 端的前 50 个氨基酸残基中,同样具有丰富的碱性氨基酸,其中有 8 个精氨酸和 6 个赖氨酸。Dasgupta 等[17]认为,这一结构特征是病毒外壳蛋白组装过程中"蛋白 - RNA"之间相互作用所必需的。

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