



Designing, cloning and amplification of pDream2.1/MCS/CII-6 recombinant plasmid which includes a mexican scorpion *Centruroides limpidus limpidus* CII-6 gene

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Abstract:

Biologically active molecules present in the venom of several species of scorpion, have shown potential against various diseases, including cancer. It has been reported that several toxins can block ion channels present in the membrane of several cancer cell lines, through action potential, altering their cell function, cell cycle arresting, and inducing apoptosis death pathway. The use of

the sequence of CII-6 gene present in the genome of the Mexican scorpion *Centruroides limpidus limpidus* encoding a beta-toxin-locker of Na⁺ channels by action potentials is proposed. This molecule has not been evaluated to determine if it has potential as an anti-cancer agent. In this paper the *in silico* design of recombinant molecule pDream2.1/MCS/CII-6, including the CII-6 gene into the polylinker site and identification by molecular biology techniques. Our results confirm a fragment of 316 base pairs, by digestion with BamH1 and Kpn1 enzymes, PCR, and the sequenced of amplicon, the alignment with CII-6 sequence reported in the GenBank database, showed 99.6% similarity and identity. The recombinant plasmid, could be used to assess potential as anti-cancer agent on several cancer cell lines.

Keywords: CII-6 toxin; *Centruroides limpidus limpidus*; pDream2.1/MCS; Cloning; Transformation; *Escherichia coli*; canal ionic; cancer

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1. Introduction

Studies with different venom toxins scorpions have shown anticancer property, to inhibit the cell proliferation, arrest the cell cycle and induce death cancer by apoptotic on several cancer cell lines (Diaz *et al.*, 2010); (Ilhem *et al.*, 2011); (Yue-Jun *et al.*, 2011, 2007); (Kievit *et al.*, 2010); (Fan *et al.*, 2010); (Gupta *et al.*, 2010); (D'Suze *et al.*, 2010); (Deshane *et al.*, 2003); (Soroceanu *et al.*, 1998, 1999).

It has been reported that the growth and invasion of several cancers is associated with dysregulation of ion channels (Le Gueneec *et al.*, 2007), a necessary mechanism that requires Na⁺, K⁺ and Cl⁻ ions to facilitate growth and invasion of a tumor cell (Mao *et al.*, 2008), therefore it is suggested that, related toxins to ion channels in the membrane cell can be used to treat certain types of cancer (Mamelak *et al.*, 2007)

A potential resource is the *Centruroides limpidus limpidus* scorpion species (CII), the second in importance in Mexico by medical reports of scorpionism (Ponce and Francke, 2004). The components of the scorpion venom are molecules physiologically active, the most important are toxins that interact selectively and specifically

with Na⁺, K⁺ (Rodriguez de la Vega and Possani, 2004; 2005), Ca²⁺ and Cl⁻ ion channel, presents in the cell membrane of mammalian, insect and crustaceans.

CII-6 is a beta-toxin of 85 aminoacids, with a molecular mass of 9,323 Daltons, CII-6 belongs to the superfamily of scorpion toxins long chain (4 C-C), inhibitors of Na⁺ ion channels, which is expressed in high concentrations and secreted in the venom gland of this scorpion, this toxin binds independently of the voltage on the site-4 of Na⁺ ion channel (NAV), and changes the voltage activation to more negative potentials, affecting the activation of Na⁺ ion channel and promoting spontaneous and repetitive shots (Coronas and Possani, 2002; Uniprot, 2016).

To date, not been explored the CII-6 molecule as anti-cancer agent, however, there are reports of toxins from the venom of different scorpions with affinity to Na⁺ ionic channels, can inhibit, control and interact with various stages of metastatic cascade, as has been evidenced in several aggressive for its high levels of expression carcinomas (Gellet *et al.*, 2009).

In the present work, is reported the *in silico* design of recombinant molecule pDream2.1/MCS/CII-6 containing the CII-6 sequence, present in the genome of *C. l. limpidus*, as well as their cloning, restriction enzyme digestion, amplification by PCR and sequencing.

2. Results

A schematic figure of pDream2.1/MCS/CII-6 is shown, is an expression vector of 7475 bp including the sequence of the gene CII-6 of 316 bp of *C. l. limpidus*, this sequence is flanked by sites for restriction enzymes EcoRI and KpnI (Figure 1).

The integrity of pDream2.1/MCS/CII-6 as DNA, is shown in figure 2B, meanwhile, enzymatic digestion of the recombinant molecule pDream2.1/MCS/CII-6 with enzymes EcoRI and KpnI shown a fragment of approximately 316 bp (Figure 2A).

The Chain Reaction of Polymerase (PCR) carried out, shown in electrophoresis, an amplified sequence of approximately 316 bp (Figure 3) and correspond to the size of the sequence for the CII-6 gene reported in GenBank with access number AF491132.

The amplicon sequenced and aligned with the sequence reported in the database GenBANK corresponding to the CII-6 gene of *Centruroides limpidus limpidus* encoding for a beta-toxin, Na⁺ channel modifier. Both show 99.6% of identity and similarity (Figure 4)

This molecule, may be used to test effect on several cancer cell lines, which is the very purpose of the investigation. It is noteworthy that the experiments were already started and show promising results.

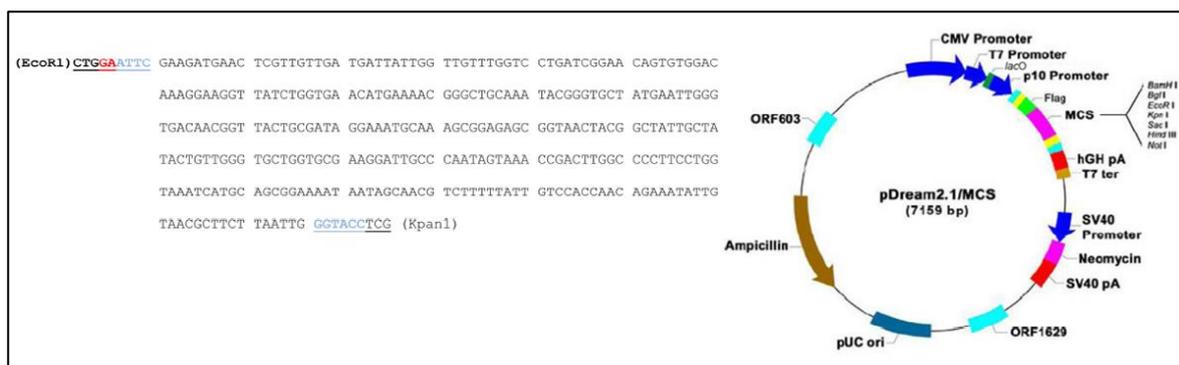


Figure 1. Design of pDream2.1/MCS/CII-6, as from the expression vector pDream2.1/MCS and sequence of CII-6 gene of *C. l. limpidus*.

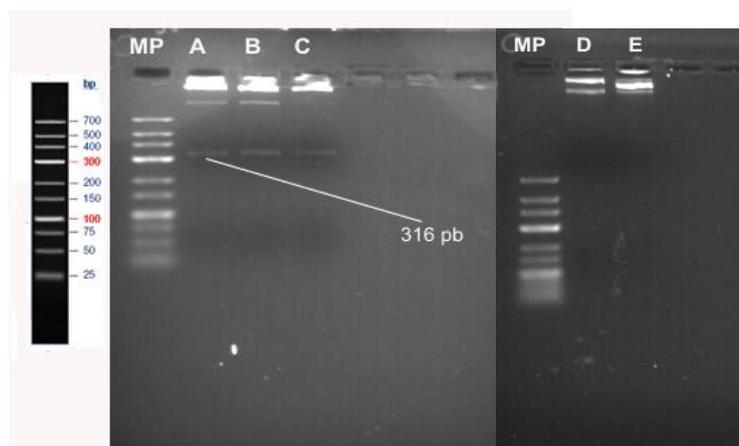


Figure 2: Electrophoresis showing a fragment of 316 bp, corresponding to the sequence of the gene CII-6 post-cloning in E. coli DH5a strain; MP: Marker weight (25-700 bp); A, B and C: Digestion of plasmid pDream2.1/MCS/CII-6 with restriction enzymes EcoR1 and Kpn1 of; D and E: plasmid DNA.

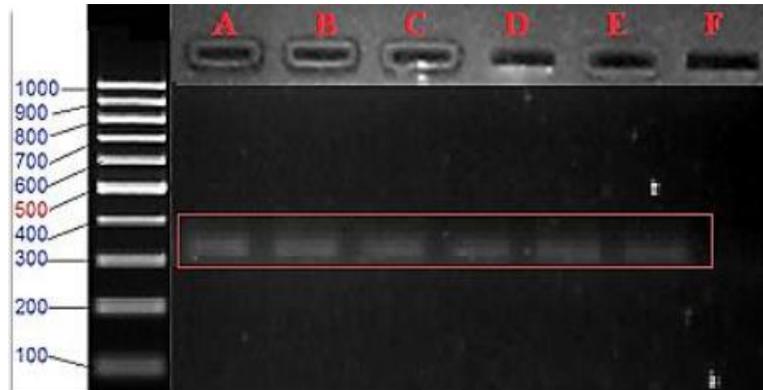


Figure 3. Electrophoresis showing amplicon of approximately 316 bp. A: molecular weight marker (100-1000 bp); B, C, D, E, F amplicons of CII-6; F: nuclease-free water.

```
#####
# Program: water
# Rundate: Wed 30 Dec 2015 20:35:45
# Commandline: water
#
# -auto
# -stdout
# -asequence emboss_water-I20151230-203543-0991-98371257-es.asequence
# -bsequence emboss_water-I20151230-203543-0991-98371257-es.bsequence
# -datafile EDNAFULL
# -gapopen 10.0
# -gapextend 0.5
# -aformat3 pair
# -snucleotide1
# -snucleotide2
# Align_format: pair
# Report_file: stdout
#####

#=====
#
# Aligned_sequences: 2
# 1: EMBOSS_001
# 2: EMBOSS_001
# Matrix: EDNAFULL
# Gap_penalty: 10.0
# Extend_penalty: 0.5
#
# Length: 250
# Identity:      249/250 (99.6%)
# Similarity:    249/250 (99.6%)
# Gaps:          0/250 ( 0.0%)
# Score: 1241.0
#
#
#=====

EMBOSS_001      1 GAAGATGAACTCGTTGTTGATGATTATTGGTTGTTGGTCCTGATCGGAA
  |||
EMBOSS_001      1 GAAGATGAACTCGTTGTTGATGATTATTGGTTGTTGGTCCTGATCGGAA

EMBOSS_001     51 CAGTGTGGACAAAGGAAGGTTATCTGGTGAACATGAAAACGGGCTGCAAA
  |||
EMBOSS_001     51 CAGTGTGGACAAAGGAAGGTTATCTGGTGAACATGAAAACGGGCTGCAAA
```

Figure 4. Alignment of sequence CII-6 and sequence GenScript by EMBOSS software.

3. Materials and Methods

Design of pDream2.1/MCS/CII-6

Was designed *in silico* the recombinant molecule pDream2.1/MCS/CII-6, incorporating into the multicloning site of commercial expression vector pDream2.1/MCS (GenScript, USA), the sequence of CII-6 gene (GenBank : AF491132) flanked by two sites for restriction enzyme EcoR1 and Kpn1, it was sent to company GenScript, for synthesis. The product freeze-dried was resuspended in 200 µl of nuclease-free water at 42 ° C.

Preparation of competent cells

Bacteria frozen Escherichia coli strain DH5-α was inoculated into 20 ml of LB medium (Luria-Bertani, Sigma SLBB9243) without antibiotic and incubated for 16 hours at 37 ° C with constant stirring (200 rpm), later, 1 ml of bacterial growth was cultured in a Falcon tube of 50 ml (Axygen Scientific, Cat. 431621) containing 10 ml of LB medium and incubated for 4 hours at 37 ° C with stirring, and was measured optical density in a spectrophotometer (Epoch: 1209284) at 450 nm.

The bacterial culture was transferred to two 50 ml Falcon tubes prechilled and centrifuged at 3000 rpm x 10 min at 0 ° C, the supernatant was decanted, and the pellet was resuspended in 1 ml of cold CaCl₂ (0.1M), the tubes were centrifuged at 3000 rpm x 10 min at 0 ° C. Subsequently was added again 1 ml of cold CaCl₂ (0.1M) and aliquoted into sterile vials precooled (Sambrook and Fritsch, 1989).

Transformation of competent cells by heat shock

Was added 1 µl of recombinant plasmid pDream2.1/MCS/CII-6 to one vial of competent cells and it was incubated for 30 minutes on ice placed, immediately in water bath at 42 ° C for 45 seconds, finally in ice for 2 minutes. Was added 900 µl of LB medium and was incubated for 1 hour at 30 ° C with stirring (220 rpm) (Sambrook and Fritsch, 1989).

Cloning and replication of Escherichia coli strain DH5-α/pDream2.1/MCS/CII-6

30 µl of cells transformed, were grown in dishes LB-agar with ampicillin (100 mg/ml, PentrexilR Bristol-Myers Squibb) and incubated at 37 ° C for 16 hours (Sambrook and Fritsch, 1989). A colony from plate with transformed bacteria growing, was inoculated into 5 ml of LB medium with ampicillin and incubated at 37 ° C for 16 hours with constant stirring.

Extraction and purification of pDream2.1/MCS/CII-6

The extraction of plasmid DNA was performed with the kit GeneJET Plasmid Miniprep (Thermo Scientific Cat. # K0502) following the manufacturer's instructions. Briefly, Escherichia coli strain DH5-α/pDream2.1/MCS/CII-6 after 16 hours in culture, was centrifuged at 3000 rpm for 10 minutes and the supernatant was discarded, the pellet was suspended with 250 µl of the solution resuspension and transferred to a sterile vial, then, were added 250 µl of Lysis Solution and mixed by inversion 5 times, after, were added 350 µl of Neutralizer Solution and again mixed by inversion. The tube was centrifuged at 13,500 rpm for 5 minutes and the supernatant was transferred to a GeneJET column, and centrifuged at 12,500 rpm for 1 minute, the filtrate was discarded, subsequently, 2 washes were performed, with 500 µl of Wash Solution and centrifuged for 1 minute at 13,500 rpm. The filtrate was discarded and one additional step of drying by centrifuging for 1 minute was performed. Finally, the column was transferred to a new microcentrifuge tube and 50 µl Elution Buffer was added, allowed to incubate for 5 minutes at room temperature and centrifuged for 2 minutes at 14,000 rpm. The extraction yield of DNA was quantified in spectrophotometer (Nanodrop Quawell 5000) at 600 nm.

Enzymatic digestion and electrophoresis

Double digestion of pDream2.1/MCS/CII-6 was performed, mixing 12.8 µl of nuclease-free water (Cat. R0581 Thermo Scientific), 2 µl of M_uLTI-

CORE Buffer (Cat. R999A, PROMEGA), 0.5 µl of restriction enzyme EcoR1 (Cat. R601A, Promega), 0.5 µl of restriction enzyme Kpn1 (Cat. R634A, Promega) and 4 µl of plasmid DNA, and incubated for 1 hour at 37 ° C, later, the fragment was visualized by electrophoresis (Sambrook and Fritsch, 1989).

PCR of pDream2.1/MCS/CII-6

For amplification of the gene CII-6, primers were used:

CII-6/F
5'CTTCTACTTGAGCAACAATA3' and

CII-6/R: 3'CAATTAAGAAGCGTTACAATA5'
a Master Mix as follows: 12.5 µl of PCR Master Mix (Promega, Cat M7502), 10 µM of each primer (10 µM), 5 µl of DNA and 4.5 µl of nuclease-free water. The reaction was performed in a thermocycler (Techne TC-512 USA) with the following parameters: Initial denaturation: 95 ° C for 5 min; 95 ° C for 30 seconds; 35 cycles at 40 ° C for 30 seconds; initial extension at 74 ° C for 30

seconds and final extension at 74 ° C for 5 minutes, finally cooling at 4 ° C for 5 minutes. The amplicons were visualized by electrophoresis.

Sequencing of amplicons (CII-6)

The PCR products were sequenced by the Sanger method by the company Genscript (Genscript Corporation, Piscataway, NJ, USA;) the received sequence was aligned with MEGA program (6.06) with the sequence CII-6 gen of the scorpion *Centruroides limpidus limpidus* reported in GenBank.

4. Conclusions

The design and assembly of pDream2.1/MCS/CII-6, were performed correctly, was verified a fragment of 316 bp approximately by digested with restriction enzymes after cloning. PCR and sequencing of amplicons, confirm the insert of CII-6 gen of *C. l. limpidus*, such molecule therefore can be used for various experiments.

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Author Contributions

JMECO: Design of pDream2.1/MCS/CII-6, write; MAEG: cloning and transformation; MCRA: Extraction of DNA, Enzymatic digestion; EGD: PCR and Sequence analysis; ABP: Design of pDream2.1/MCS/CLL-6, Suggestions and article revision; JCVC: Suggestions and article revision.

Conflicts of Interest

The authors declare no conflict of interest

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