OL-PCR for Site-directed Mutagenesis of Full-length cDNA of DEN-2

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Abstract: Aim. To generate the oligonucleotide-directed mutants of the full length cDNA clone of dengue 2 virus. **Methods.** Two DNA fragments with single point mutation (E62 or E203) were amplified with four pairs of oligonucleotide primers by OL-PCR and then cloned into pGEM-T vectors respectively. The recombinant T vectors, TB62 and TB203, were digested with *Cla* I + *Sph* I and *Sph* I + *Nhe* I, then ligated to pDVWS501 with T4 DNA ligase respectively. The recombinant plasmids, TB62 and TB203, were sequenced. **Results.** The results of DNA sequencing indicated that TB62 and TB203 with point mutation were obtained. **Conclusion.** OL-PCR could be applied to site-directed mutagenesis of large plasmid (>16 Kb). [Life Science Journal. 2006;3(3):53-57] (ISSN: 1097-8135).

Keywords: dengue 2 virus; full-length cDNA clone; site-directed mutagenesis; OL-PCR

1 Introduction

The site-directed mutagenesis *in vitro* to target DNA sequence is a common-used way of molecular biology, but it is still a big problem to mutagenesize a large plasmid at present. What researchers always explore, under the operated conditions, is to maintain the sequences other than the sites of mutation intact. In this research, by using the technique of OL-PCR, the site-directed mutagenesis to a large plasmid of 16 kb has been established successfully. According to the sequenced results, there has been a consistence between the sequences of the mutagenesized plasmid and the designed ones.

By comparing the amino acids sequences of protein E of 3 dengue virus strains in our laboratory, 2 loci discovered, which locate in site 62 and 203 of protein E respectively, may be relevant to suckling mice neurovirulence. When the E62 is Glu and the E203 is Asp, the virulence is decreased; While the E62 is Lys and the E203 is Trp, the virulence is increased. pDVWS501 is a plasmid containing the full-length cDNA of D2-MON501, and RNA *in virto* transcripts from pDVWS501 could be recovered to infectious virus (MON501) upon electroporation into BHK cells. Then, the infected suckling mice with MON501 appeared to be the encephalitis symptom. By OL-PCR, we want to mutagenesize the amino acids of E62 and E203 in pDVWS501, and then mutated MON501 would not show neurovirulence on suckling mice. In a word, we hope this research would lay a base for further study on the influence of the 2 amino acids upon suckling mice neurovirulence.

2 Materials and Methods

2.1 Strains and plasmids

The pDVWS501 is a plasmid which contains the full-length cDNA clone of DEN-2, and this plasmid was kindly provided by Dr. Wright of Monash University in Australia. AF038403 is the Accession Number of complete genome sequence of D2-MON501. The *E. coli* DH5a was stored in our laboratory and the clone vector pGEM-T was purchased from Promega Company.

2.2 Enzymes and other reagents

The restriction enzymes were purchased from Biolab Company. The T4-DNA ligase was purchased from Promega Company. The IPTG and X-gal were purchased from Huamei Biology Engineering Company. The Pwo DNA polymerase, expand high fidelity PCR system and DNA recoverying kit were provided by Boeringer Mannheim Company. QIAGEN plasmid midi kit was purchased from QUIAGEN Company.

2.3 Primers

The primers were designed with the DNAstar's Quickpri software package and synthesized by Sangon Co. Ltd. The New3 and New4

hold the E62 site with point mutation (GAG→AAA). The New5 and New6 carry the E203 site with point mutation (AAT→GAC). In Table 1, the italics with underlines in the primers sequences are the ones after point mutagenesis.

Table 1. Oligonucleotide primers used for amplification

Primer name	Genome position*	Primer sequence(5′→3′)
New-1	1638-1656	CGGGCCTCTTCGCTATTAC
New-3	2968-2992	GGTCAGCTTTGC TTTTATACAGTAC
New-4	2968-2992	GTACTGTATA AAAGCAAAGCTGACC
New-7	3026-3042	GGAGAACCCAGCCTAAA
New-5	3391-3413	GCCAAGCTTT GTCTTCCATTTGC
New-6	3391-3413	GCAAATGGAA GACAAAGCTTGGC
R1566	3400-3423	CTGTGCACCAGCCAAGCTTTATTT
New-2	4388-4411	TGAAGCTAGCTTTGAAGGGGATTC

* Genome positions are given according to the sequence of pDVWS501

2.4 Plasmid PCR

With pDVWSK501 as templates and New1, New3, New4, R1566, New7, New5, New6, New2 as primers, we've amplified the segments which were named as pm1, pm2, pm4 and pm5 by PCR. These segments have been purified by DNA recovery kit.

PCR reaction system: 10 mmol/L dNTPs 0.6 μl, upstream primer 1.5 μl, downstream primer 1.5 μl, plasmid as template (about 10 ng/μl) 1 μl, 10× buffer 3 μl and water 22 μl. After denaturation of 2 min at 94 ℃, we added 0.5 μl Pwo DNA Polymerase into the PCR reaction system, then starting PCR cycling. Reaction parameters are as followed: ① pm1 and pm4:94 ℃ for 15 sec, 55 ℃ for 30 sec, 72 ℃ for 1.25 min, after 25 cycles, 72 ℃ continuously lasted for 7 min; ② pm2 and pm5:94 ℃ for 15 sec, 55 ℃ for 30 sec, 72 ℃ for 45 sec, after 25 cycles, 72 ℃ continuously lasted for 7 min.

2.5 OL-PCR

After making electrophoresis with 1% agarose gel and estimating the content of pm1, pm2 pm4 and pm5, we diluted the four segments to 0.5 ng/μl, respectively and then mixed pm1 and pm2 together, so did pm4 and pm5. PCR reaction system: 10 mmol/L dNTPs 0.6 μl, templates (pm1 and pm2 or pm4 and pm5) 2 μl, 10× buffer 3 μl and 21.9 μl water. After denaturation of 2 min at 94 ℃, 0.25 μl expand high fidelity DNA polymerase was put into the above mixture, then followed PCR cycling. Reaction parameters: 94 ℃ for 15 sec, 55 ℃ for 1 min, 72 ℃ for 2 min, after 3 cycles, 72 ℃ lasted for 5 min. After the primer New1 1 μl and R1566 1 μl or New7 1 μl and New2 1 μl were respectively added into the PCR mixture above and denatured of 2 min at 94 ℃, 0.25 μl expand high fidelity DNA polymerase was added into the before system then running the second PCR cycles. Reaction parameters: 94 ℃ for 15 sec, 55 ℃ for 1 min, 72 ℃ lasting for 3 min, after 25 cycles, 72 ℃ continuously kept for 7 min.

2.6 Constitution of plasmid T-TB62 and T-TB203

After purifying the pm3 and pm6 fragments obtained by OL-PCR, according to the specification of pGEM-T vector system kit, the two segments were ligated to pGEM-T vectors and transformed into DH5a with these recombiant plasmids, and the positive clones of T-TB62 and T-TB203 were selected. We identified the recombinant plasmids by digesting them with endorestriction enzymes and ran the PCR. The recombinant plasmids were sequenced with automatic-sequenator of ABI377 version.

2.7 Constitution and clone of plasmids TB62 and TB203

T-TB62 was digested with *Cla* I + *Sph* I for 2 h, therefore, we've got the segments tb62 about 1463 bp through purifying. Another plasmid T-TB203 was digested with *Sph* I + *Nhe* I for 2 h and then tb203 about 1167 bp was purified. Plasmid pDVWS501 was mono-digested by *Cla* I for 3 h and then being purified. The purified segments were mono-digested again with *Sph* I for 3 h and then the longer segments ZT-203 were purified. pDVWS501 plasmid was digested with *Sph* I + *Nhe* I at the same time for 1.5 h. In order to digest the pDVWS501 completely, we added *Sph* I + *Nhe* I into the previous mixture once again and lasted for another 1.5 h. Consequently the longer fragment ZT-203 was acquired and purified. The puri-

fied segment ZT62 was amplified by PCR with New7 and New5 as primers and another purified segment ZT203 was amplified with New4 and R1566 as primers. If the results were negative, the following proceure would be done.

Tb-62 and ZT-62, tb-203 and ZT-203 were respectively diluted to 3:1 in moles and ligated with T4 DNA ligase. With the recombinant plasmids before, DH5a was transformed again and the positive clones were selected. TB62 and TB203, the full-length cDNA clone of DEN-2 with point mutation should be tested for their correctness with digestion and plasmid PCR.

2.8 Sequencing

Plasmids TB62 and TB203 were extracted with QIAGEN plasmid midi kit and sequenced with automatic-sequenator of ABI377 version.

3 Results

The establishment of the method to dengue 2 virus full-length cDNA site-directed mutagenesis bases on the facts that, in the plasmid pDVWS501 with the cDNA, both ends of the E62 have mono-digestion sites of *Cla* I and *Sph* I; both ends of the E203 have mono-digestion sites of *Sph* I and *Nhe* I.

3.1 Constitution and clone of plasmid T-TB62 and T-TB203

Firstly with pDVWS501 as templates 2 groups of short segments were amplified: pm1 and pm2, pm4 and pm5, which were 1355 bp, 456 bp, 1021 bp, 388 bp in length, respectively and partly overlapped one another. Then pm1 and pm2, pm4 and pm5 were mixed in equal quantity separately. Long templates were acquired by the first PCR and the long segments pm3 and pm6 were obtained through the second PCR running, which were 1786 bp and 1386 bp in length, respectively (Figure 1). After pm3 and pm6 were cloned into pGEM-T vectors separately, the recombinant plasmids T-TB62 and T-TB203 were obtained. The two plasmids were transformed into DH5a. Running PCR to plasmid T-TB62 with primers New1 and R1566, a segment was about 1786 bp in length. After the two plasmids were digested with *Cla* I and *Sph* I, 2 segments of 1463 bp and 3323 bp in length separately were got. Running PCR to the plasmid with primers New7 and New2, the target segment about 1386 bp in length. After the same plasmid was digested with *Sph* I and *Nhe* I, other two target segments of 1167 bp and 3219 bp in length respectively were got (Figure 2). The sequencing results indicated that there were expected shifts only in mutated loci and the other loci were the same as before by comparing T-TB62 and T-TB203 with pDVWS501.

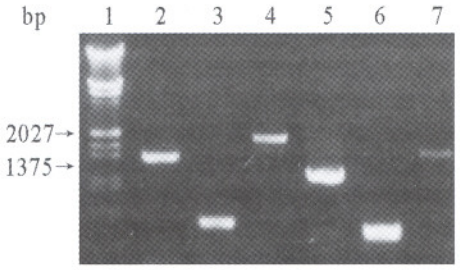

Figure 1. DNA fragments with point mutation amplified by OL-PCR
Lane 1: λDNA/*Eco*RI + *Hind* Ⅲ marker; Lane 2: pm1; Lane 3: pm2; Lane 4: pm3; Lane 5: pm4; Lane 6: pm5; Lane 7: pm6

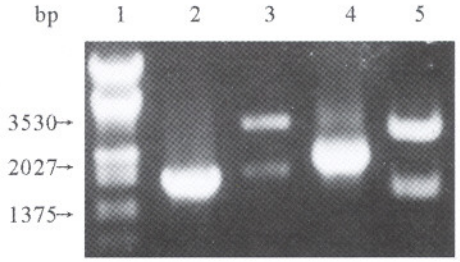

Figure 2. Identification of recombinant plasmids T-TB62 and T-TB203
Lane 1: λDNA/*Eco*RI + *Hind* Ⅲ marker; Lane 2: T-TB203 PCR assay; Lane 3: T-TB62 digested with *Cla* I + *Sph* I; Lane 4: T-TB62 PCR assay; Lane 5: T-TB203 digested with *Sph* I + *Nhe* I

3.2 Constitution and clone of plasmids TB62 and TB203

After pDVWS501 and T-TB62 were respectively digested with *Cla* I and *Sph* I and pDVWS501 and T-TB203 were respectively digested with *Sph* I and *Nhe* I, the same adhesive-ends of vectors and inserting-segments were got. After the inserting-segments were ligated to the vectors *in vitro*, the recombinant plasmids were transformed into DH5α and later the positive clones were screened with LB/Amp(+) plates. Recombinant plasmids were extracted from the picked positive clones and were mono-digested with *Cla* I, then a segment of 16151 bp in length were got. We've run PCR to plasmid TB62 with primers New6 and New2 and obtained another segment of 1356 bp. After TB203 was mono-digested with *Nhe* Ⅰ, a segment was got, which was 16151 bp in length. PCR were run to TB203 with New6 and New2 as primers, and a fragment of 1021 bp were acquired. All of the work above might show the success in constituting the full-length cDNA clone TB62 and TB203 of dengue 2 virus with point mutation (Figure 3).

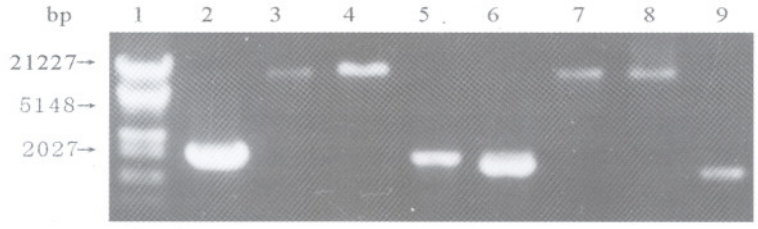

Figure 3. Identification of recombinant plasmids TB62 and TB203
Lane 1: λDNA/*Eco*RI + *Hind* Ⅲ marker; Lane 2: purified tb62; Lane 3: purified ZT62; Lane 4: TB62 digested with *Cla* I; Lane 5: TB62 PCR assay; Lane 6: purified tb203; Lane 7: purified ZT203; Lane 8: TB203 digested with *Nhe* I; Lane 9: TB203 PCR assay

3.3 Sequencing

The results of sequencing indicated that we've succeeded in constituting the full-length cDNA clones TB62 and TB203 with point mutation in sites 62 and 203 of protein E in plasmid pDVWS501.

4 Discussion

As for site-directed mutagenesis on the DNA segments larger than 1 kb, it is the most difficult problem to keep high fidelity. Among the DNA polymerase, T4 DNA polymerase, owing to its strong exonuclease activity of single and double-strand DNA 3′→5′, is the most suitable candidate for site-directed mutagengsis *in vitro*.

There are many kits for site-directed mutagengsis on plasmids. But some of them need particular vectors, which therefore depend on convenient restriction endonuclease sites in the mutated DNA segments; some of them utilize vectors designed by researchers. All of these kits are designed by similar principles that after synthesizing all sequences of the target plasmid *in vitro* by T4 DNA polymerase and ligating them together with T4 DNA ligase, the characteristic shifts of plasmid, which were easily picked out, are used as screening indicators. So the larger the plasmid is, the more difficult it is that mutagenesized-plasmid is obtained and the fidelity to those sequences except for mutation loci is guaranteed.

The aim of this research was to solve the problem of site-directed mutagenesis to plasmid pDVWS501 which is about 16 kb in length. By comparing this plasmid's zymogram, we discovered that there separately was a mono-restriction enzyme site nearby the ends of the 2 mutagenesis loci, which were 1463 bp and 1167 bp in distance respectively. But all of these restriction enzyme sites were not suitable for the point mutagenesis kits of Promega Company and the plasmid itself was beyond the applying confines of point mutagenesis kits. So, we selected 2 kinds of DNA polymerase and adopted the method of OL-PCR in this research. Pwo DNA Polymerase, whose accuracy is 10 times stronger than that of Taq DNA Polymerases, is of strong 3′→5′ exonuclease activity and more than this, its PCR product has no "A" on its 3′ ends – that are blunt ends, which avoids the mismatch and disturbance to the reaction of OL-PCR. Expand™ High Fidelity Sys is of part 3′→5′ exonuclease activity, therefore, the PCR product is the mixture with the blunt ends and 3′ ends with "A", which can be conveniently cloned into pGEM-T vectors. As is stated above, with the peculiarity of these 2 kinds of polymerase in our research, we've enhanced the fidelity to OL-PCR as possible as we could, and hence paved the way for ligating T-vectors. With expand high fidelity PCR system, if 20 effective cycles would be carried out to amplifying the segments of 1 kb, there were 92% segments identical to the templates in theory. Although the accuracy rate of these 2 kinds of DNA polymerase was lower than that of T4 DNA polymerase, owing to the amplified segments being greatly shortened *in vitro*, it still relatively lowered the error rate during replication. In this experiment, 2 clones were selected separately from T-TB62 and T-TB203 for sequencing and the expected results were got.

In the course of site-directed mutagenesizing designed by this research, there was a biggest shortcoming that was, except for the directly sequencing, no other simple ways for identifying the plasmid before and after its mutagenesizing. So, there may be false-positive results in the latter work when digesting the vectors were incompletely. In order to avoid the false-positive results, the restriction endonuclease was applied, intentionally extended the time of digestion (3 h), then another time restriction endonuclease was added at the midterm (1.5 h) which could digest the plasmid completely. After digested and purified, the plasmid was identified by PCR. The negative result proved the complete digestion of plasmid, which

would perfect the whole course of experiment and secured the comparatively high mutation rate. At last, TB62 and TB203 were transformed into DH5a and from this transformation 6 clones were picked out for identification of PCR and digestion. Among which, there were 5 and 4 positive clones respectively, and from these positive ones, 2 clones were respectively chosen to determinate their mutation sites. As a result, expected mutation sites were finally obtained.

The method of site-directed mutagenesis established by us suits for all successfully constituted plasmids. But the only limitation of this method is the 2 ends of the sites of point-mutagenesizing must have mono-digestion sites. If the amplified segments by OL-PCR were smaller than 2 kb, there were relatively high fidelity and mutation efficiency in the process of mutagenesizing.

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