RECOVERY OF ANTIBIOTIC RESISTANCE GENES IN NATURAL ENVIRONMENTS

Mai Thi Ngoc Lan Thanh[(1)], **Le Phi Nga**[(2)]

(1) Thu Dau Mot University; (2) University of Science (VNU-HCM)

ABSTRACT

Recently environmental metagenomics are useful methodology to study microbial diversity in the environment as well as functional metabolic genes. This study was also based on metagenomic method to discover antibiotic resistance genes from aquatic environments. To create a metagenomic library, the environmental DNA was extracted from water and sediment sample of Thi Nghe canal, Ho Chi Minh City. Total DNA then was fragmented by sizes of 1-3 kb and inserted in to pUC19 plasmid. After transformation into E.coli DH5α host, transfomants were screened by growth on a minimal inhibition concentration (MIC) of antibiotics. Results showed that antibiotic MIC values for Ecoli DH5α/pUC19 used as a negative control are 5μg/ml gentamicin, 6μg/ml chloramphenicol, and 50μg/ml streptomycin and 30μg/ml tetracyclin. From a newly created environmental DNA library of 1.315 mega bases (337 transformants) 176 clones resistant to gentamicin and 284 clones resistant to chloramphenicol were found, but either recombinant resistant to streptomycin nor to tetracycline. Because of timing limited for a Msc. study, the sequences of clones have not been verified yet. However, primarily results showed here indicate that the antibiotic resistant gene(s) from an aquatic environment in Ho Chi Minh city could be cloned for further studies.

Key words**:** *environmental metagenomic, antibiotic resistance genes, uncultured microorganism*

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1. Introduction

It has been estimated that less than 1% population of microorganisms in our earth are cultivable, especially, only 0.1% known in marine environment[27]. Many useful microorganisms have being used in the industry and environment. Microbes are powerful bioconversion "machines" that play important roles in degradation of natural as well as synthetic compounds including drugs or antibiotics, thus many of them are antibiotic resistant.

Metagenomics, the genomic reconstruction from environmental samples can be a pool for accessing the untapped resources of microbial biodiversity, which was larger than that seen by traditional methodologies [9-12, 13-15]. Recently some functional genes such as synthesis of biocatalysts, enzymes, antibiotic and antibiotic resistance genes have been reported from metagenomic libraries.

Antibiotic resistance genes are generally cloned by a targeted PCR from a cultivable microorganism. This method can not assess the major uncultivable population of microorganisms that is believed to be more than 99%[4,5,6,7,8], thus novel

antibiotic resistance genes are still under recovered[1].

The polymerase chain reaction (PCR) can be used for cultureindependent isolation of antibiotic resistance genes from environmental samples [16-20]], but only accesses genes that are similar to known sequences and often does not recover complete genes. Here we circumvented the limitations of both culturing and PCR based methods by extracting total DNA directly from environmental samples and cloning it, thus constructing libraries include the genes of uncultured microorganisms[1]. Clones exp-ressing various enzymes reported previously [22][21][23] were from environ-mental metagenomic libraries [21].

To construction of a metagenomic library, several vectors have being used such as Fosmid vector [29], Cosmid [1], BCA vector [28], or plasmids [1] and the host can be *E.coli* [28],[29],[1] or *Pseudomonas* sp [30] depending on purposes. This study was based on construction of a metagenomic library using plasmid pUC19 and host *E.coli* DH5α. The environment site for study is Thi Nghe bridge that belongs to Thi Nghe canal in Ho Chi Minh city.

For screening antibiotic resistant *E.coli* strains bearing recombinant pUC19 plasmids, 4 common antibiotics such as gentamicin, tetracycline, chloramphenicol, streptomycin were used.

2. Experimental procedure

2.1. Materials and chemicals

Wizard® SV Gel Kit and PCR Clean-Up System (Promega) were purchased from Promega. Antibiotics were from HCMC Food Drug Quality Control Institute. Restriction enzymes were products of Invitrogen. All other chemicals used were highest purity.

The *E.coli* strain DH5α (*F⁻ø80dlac-ZΔM15 Δ(lacZYA-argF) U169 deoR recA1 endA1 hsdR17(rₖ⁻, mₖ⁺) phoA supE44 λ⁻ thi-1 gyrA96 recA1*) (Life Tech-nologies) was used as the host strain for maintaining libraries. Strains were grown in LB- medium with 100µg.ml^{-1} Amp and if it is necessary, an appropriate antibiotic was added.

2.2. Sampling and samples storage:

Each time, 5 litters of canal bottom water containing top-layer of sediment samples was collected from Thi Nghe canal. Samples were immediately transferred to the laboratory and centrifuged at 12,000 rpm 4^{o}C for 10 min. Cell pellet was immediately under step of extraction of total DNA or stored under – 80^{o}C for later use.

2.3. Determination of MIC (Minimal Inhibition Concentration) of E.coli DH5 a/pUC19.

Minimum inhibitory concentrations (MICs) were determined using microtitre plate dilution assays in LB broth with the various concentrations of each of 4 antibiotics. The lowest concentration of antibiotic at which *E.coli* DH5α/pUC19 does not growth is defined as a MIC.

2.4. Extraction of total DNA from environmental samples.

Total DNA from pellet containing cells was extracted by manual protocol. In that protocol, pellet (from about 1 liter sample) was re-suspended by 200µl solution (Tris-HCl pH 8.0) and then 5.5µl protease K and 15µl 20% SDS added and mixture was incubated for an hour at 37^{o}C. After that 30 µl CTAB and 30 µl 5M NaCl were added and mixture was further incubated at 65^{o}C

for an hour. The treated sample was extracted three times with same volume of P:C:IAA mixture. Each time, after 10 min shaking by hands mixture was centrifuged at 14,000 rpm for 5 minutes. The supernatant finally was precipitated with 2.5 volume of ice-cold 96% ethanol and 1/10 volume per volume of 3M CH_3COONa, pH 4,5, and stayed at -20^{o}C for 15-20 minutes. Total DNA pellet after collected by centrifugation was air dried and re-suspended by 50 µl TE buffer.

2.5. Construction of recombinant pUC19 caring inserted DNA fragment from environment samples.

Total DNA was digested with 3 pairs of the restriction enzymes: HindIII - EcoRI; HindIII - KpnI; or HindIII – BamHI, respectively. DNA fragments from 1- 3kb were cut out and purified by kits and then inserted into the same restriction enzymes sites (multicloning sites) of pUC19. The ligated mixture was transformed into *E.coli* DH5α host cell and plated onto LB-Amp agar for numeration of tranformants. The table below is the designs of ligation mixture.

Table-1: *Insertion of the fragments into pUC19 vector:*

Tube	HindIII-EcoRI	HindIII-KpnI	HindIII-BamHI
DNA fragment	6µl	6µl	6µl
pUC19 vector	6µl	6µl	6µl
Ligation buffer 10X(with ATP at 10mM)	2µl	2µl	2µl
H_2O	6µl	6µl	6µl
T4 DNA ligase (3U/ml)	2µl	2µl	2µl

2.6. Screening transformants for antibiotic resistance clones.

Transformant were replicated on to LB-Amp and LB-Amp containing an additional antibiotic with MIC: 50µg.ml^{-1} streptommycin, 30µg.ml^{-1} tetracycline, 5µg.ml^{-1} gentamycin or 6µg.ml^{-1} cloramphenicol, respectively. Plates were incubated overnight at 37^{o}C. Positive clones were verified by growth in both types of plates and in construct with the negative control of *E.coli* DH5α/ pUC19 that can only grow in LB-Amp.

3. Results

3.1. MIC values of E.coli DH5α/pUC19

The minimum inhibitory concentrations (MICs) of 4 antibiotics obtained on the E.coli DH5α/pUC19 were various from 5- 50 µg/ml depending on type of an antibiotics used. The tables below are results of MICs determination with 4 antibiotics.

MIC value of chloramphenicol is 6µg/ml, of streptomycin is 50µg/ml, of gentamicin is 5µg/ml, and of tetracycline is 30µg/ml.

Table-2a: *MIC of chloramphenicol* [(+) : growth,(-): no growth]

Chloramphenicol concentration (µg/ml)	1	2	3	4	5	6	7	8	9	10	11	12
Growth of DH5α/pUC19	+	+	+	+	+	-	-	-	-	-	-	-

Table-2b: *MIC of streptomycin* [(+) : growth,(-): no growth]

Streptomycine concentration (µg/ml)	10	15	20	25	30	35	40	45	50	55	60	65
Growth of DH5α/pUC19	+	+	+	+	+	-	-	-	-	-	-	-

Table-2c: *MIC of gentamicin* [(+) : growth,(-): no growth]

Gentamicine concentration (µg/ml)	1	5
Growth of DH5α/pUC19	+	-

Table-2d: *MICs of tetracycline* [(+) : growth,(-): no growth]

Tetracycline concentration (µg/ml)	5	10	30
Growth of DH5α/pUC19	+	+	-

2. Creation of an environmental meta-genomic

Figure-1: *From left to right lanes, DNAs extracted from sediment (lane 1) and from water (lanes 2 and 3). 2µl of 50 µl of total DNA loaded per a lane.*

The first step of making a metagenonic library from an environmental sample is total DNA extraction. In figure-1, the concentration of DNA extracted from sediment sample is higher and more smear band than that of DNA extracted from water sample. This may indicate that DNA from sediment sample is more diverse thus it is better use for purpose of mining a novel functional gene.

Environmental DNA extracted was digested by each pair of HindIII-EcoRI, HindIII-KpnI, or HindIII-BamHI. The figure-2 shows environmental DNA fragments cut by size 1-3 kb.

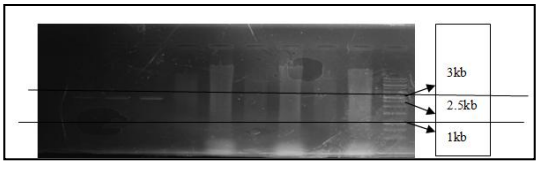

Figure-2: from right to left, DNA ladder (lane-1), sediment DNA digested by *Hind*III-*Eco*RI (lane-2), water DNA digested by *Hind*III-*Eco*RI (lane-3), sediment DNA digested by *Hind*III-*Kpn*I (lane-4), water DNA digested by *Hind*III-*Kpn*I (lane-5), sediment DNA digested by *Hind*III-*Bam*HI (lane-6), water DNA digested by *Hind*III-*Bam*HI (lane-7), pUC19 digested by either *Hind*III-*Eco*RI, *Hind*III-*Kpn*I, or *Hind*III-*Bam*HI (lanes: 8,9,10)

DNA fragments and pUC19 vector were tested to determine DNA ratio in ligation mixture.

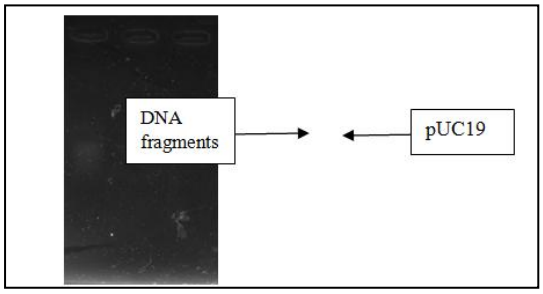

Figure-3: *Testing DNA fragments and pUC19 after extracted by kit gel extraction.*

DNA vector: fragment in ligation mixtures was 1:1 as showed in table-1, this is the best ratio giving a highest transformant counts. Results showed that for 3 ligation mixtures (3 types of digested DNA fragments total of 678 clones (table-3) were obtained. From that 17 clones were picked up to verify the insert. As it is showed in figure-4, all 17 clones carried inserts. All most plasmid had 2 bands of fragments, which are indication of a right insert. The remaining lanes showed only single bands these may due to the size of insert equals to the size of vector or the two vector was ligated together. For the higher size single band, the plasmid may be contained an insert but the restriction enzyme site were altered during ligation step.

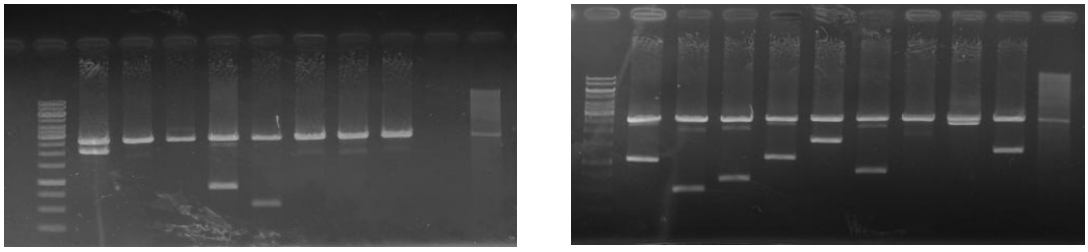

Figure-4: *Left picture: DNA ladder (lane-1), 8 transformant plasmids digested with HindIII-EcoRI (lane 2-9); pUC19 digested with HindIII-EcoRI (lane-11) Right picture: DNA ladder (lane-1), 9 transformant plasmids digested with HindIII-EcoRI (lane 2-8) by HindIII-KpnI (lane 9-10), pUC19 digested with HindIII-EcoRI (lane-11).*

Thus we have been otained 3 libraries with 1-3 kb inserts from environmental DNA. The inserted size was calculated using DNA ladder. Size of total 3 libraries was estimated as shown in table-3 yield about 1.3 mega bases.

Table 3. *Characteristics of water metagenomic library*

Library name	vector	Enzyme used for cloning	No of clones	Average insert size (kb)	Amount of cloned DNA (mega bases)
LT1	pUC19	*Hind*III and *Eco*RI	273	1.62	0.45
LT2	pUC19	*Hind*III and *Kpn*I	55	2.3	0.13
LT3	pUC19	*Hind*III and *Eco*RI	350	2.1	0.735

3. *Screening for antibiotic resistance clones*

After screening 337 transformants with each of 4 antibiotics, we found 167 clones resistant to 5 μg/ml gentamicin, and 284 clones resistant to 6 μg/ml chloramphenicol. Neither growth was found on plate containing 30 μg/ml tetracyclin nor 50 μg/ml streptomycin. 7 clones from those positive ones and re-grown in 5 μg/ml gentamicin (Fiure-5A) were checked with their plasmids for the inserts. Figure-5B shows among 7 clones 5 had inserts (lanes 2, 4, 5, 6, and 7). 2 others ones were non-specific inserts.

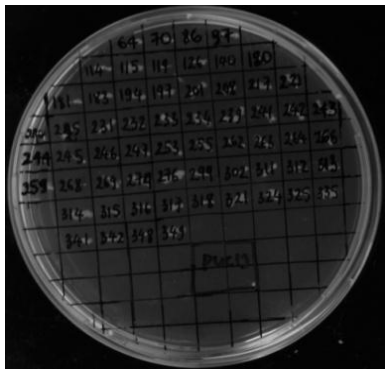

Figure 5A: *Testing the expressing resistance antibiotic of specific clones (167/337). DH5α/pUC19 is negative control on LB-Amp/gentamycin(5μg.ml^{-1})*

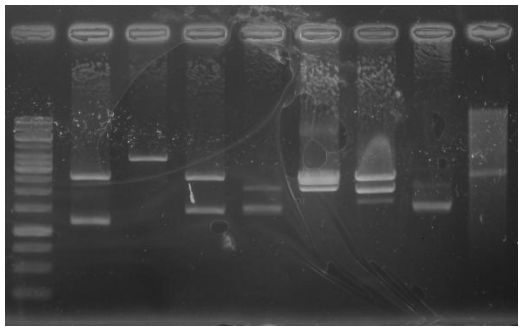

Figure 5B: *Testing plasmid of gentamicin resistance from left to right, DNA ladder(lane1), clones HE239(lane 2), HE243(lane 3), HE263(lane 4), HE264(lane 5), HK312(lane 6), HK313(lane 7), HK325(lane 8), HE/pUC19(lane 9).*

4. Discussion

Metagenomic analysis has advantages over cultivation or PCR-based methods for isolating antibiotic resistance genes because of several reasons below [1]:

− provides access to uncultured mic-roorganisms,

− does not require prior knowledge of gene sequences,

− recovers complete genes.

Although having several advantages as above, in this study, we have realized that the first difficulty is to obtain the high purity of the total DNA extracted from an environmental sample. This DNA often contain un-purity substances thus interferer with enzymatic reactions. The second difficulty is a suitable expression system for an interest functional gene. The third is that working with antibiotic resistance strains defined by its growth on MIC –agar plate, however, the growths may include artifact from contaminated ones.

The result here with 50% and 84% of transformants were resistant to gentamicin and chloramphenicol, respectively, are abnormal high frequencies. We do not have any suitable explanation for these at this time point. The plasmids of positive antibiotic resistant must be verified by sequencing and compare with known sequences. Once sequence of genes were verified we can further studied in which way the resistance was done.

5. Conclusion

The aim of study was to clone the antibiotic resistance genes from environ-mental DNA has been archived for gentamicin and chloramphenicol. Obtained *E.coli* DH5α clones expressed antibiotic resistance properties on agar plates, but their recombinant plasmids have not been further verified by DNA sequencing. This work has contributed to the type of study on a functional gene from a metagenomic library.

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THU NHẬN CÁC ĐOẠN GEN KHÁNG SINH TỪ MÔI TRƯỜNG TỰ NHIÊN

Mai Thị Ngọc Lan Thanh(1), **Lê Phi Nga**(2)

(1) Trường Đại học Thủ Dầu Một, (2) Trường Đại học Khoa học Tự nhiên (VNU-HCM)

TÓM TẮT

Gần đây, thư viện gen thuộc về môi trường hữu dụng cho các phương pháp nghiên cứu đa dạng vi sinh vật trong môi trường cũng như các gen có chức năng trao đổi chất. Nghiên cứu này dựa vào phương pháp thư viện gen để khám phá ra những gen kháng kháng sinh từ môi trường nước. Để tạo ra được một thư viện gen, DNA được tách từ mẫu nước và mẫu bùn của kênh Thị Nghè (thành phố Hồ Chí Minh). DNA tổng sau đó cắt thành những đoạn có kích thước từ 1-3kb và sau đó những đoạn DNA này sẽ được chèn vào plasmid pUC19. Sau khi chuyển gen vào tế bào E.coli DH5α, những tế bào chuyển gen được khảo sát sự phát triển trên môi trường bổ sung nồng ức chế tối thiểu của kháng sinh. Các kết quả chỉ ra rằng giá trị nồng độ ức chế tối thiểu của kháng sinh dành cho chủng Ecoli DH5α/pUC19 được sử dụng như đối chứng âm là 5μg/ml gentamicin, 6μg/ml chloramphenicol, 50μg/ml streptomycin và 30μg/ml tetracyclin. Từ thư viện DNA môi trường mới với kích thước 1.315

Mb (337 dòng tế bào chuyển gen) có 176 dòng kháng gentamicin và 284 dòng kháng chloramphenicol được tìm thấy, nhưng không có các chủng tái tổ hợp nào kháng với streptomycin và tetracycline. Bởi vì giới hạn thời gian của một luận văn thạc sĩ, nghiên cứu giải trình tự gen của những dòng kháng kháng sinh đã không được thực hiện. Tuy nhiên, các kết quả chỉ ra rằng các gen kháng kháng sinh từ môi trường nước ở thành phố Hồ Chí Minh đã được tạo dòng cần phải được nghiên cứu nhiều hơn.

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