ยีนดื้อยาในกลุ่มเบต้าแลคแทมในน้ำทิ้งของโรงพยาบาลแห่งหนึ่ง

Beta Lactamase Gene in Hospital Effluent

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บทคัดย่อ

ปัจจุบันแบคทีเรียดื้อยานับเป็นภัยคุกคามต่อการสาธารณสุข โรงบำบัดน้ำเสียของโรงพยาบาลจัดเป็นแหล่งสะสมปัจจัยต่างๆ ที่เกี่ยวข้องกับการดื้อยาปฏิชีวนะที่สำคัญ วัตถุประสงค์ของการศึกษานี้เพื่อตรวจหายีนควบคุมการสร้างเบต้าแลคแทมเมสในน้ำทิ้งจากโรงบำบัดน้ำเสียของโรงพยาบาลแห่งหนึ่ง โดยการสร้างเมต้าจีโนมิคพลาสมิดไลบราลี จากนั้นคัดเลือกโคลนที่ดื้อต่อยาในกลุ่มเบต้าแลคแทมและมีขนาดเฉลี่ย >20 กิโลเบสจำนวน 30 พลาสมิดไปวิเคราะห์ ผลการตรวจพบว่าทั้ง 30 โคลนมี bla_{TEM} ข้อมูลจากการศึกษานี้แสดงให้เห็นอย่างชัดเจนว่าแบคทีเรียที่มีพลาสมิดดื้อยาสามารถหลุดรอดจากขบวนการบำบัดน้ำเสียของโรงพยาบาลได้

คำสำคัญ: *เบต้าแลคแทมเมสยีน พลาสมิด โรงบำบัดน้ำเสียของโรงพยาบาล*

Abstract

Antibiotic resistant bacteria are now a public health threat. Hospital wastewater treatment plants (WWTPs) are particularly significant reservoirs of antibiotic resistance determinants. The aim of this study was to detect beta-lactamase genes from the final effluent of a hospital WWTP. A metagenomics plasmid library was constructed and 30 beta-lactam resistant clones with average sizes >20 kb were selected for characterization. The

results revealed that all of the 30 clones carried bla_{TEM}. The data clearly shows that bacteria harboring antibiotic resistant plasmids have evaded the treatment processes.

Keywords: *beta-lactamase genes, Plasmids, Hospital wastewater treatment plant*

1. Introduction

Antibiotic resistant microorganisms are now become an important public health problem in all over the world (Rosenthal et al., 2012). In Thailand, the problems of antibiotic resistant bacteria have been reported with increasing rates (Apisarnthanarak et al., 2009; Apisarnthanarak and Mundy 2009; Mootsikapun et al., 2009). Antibiotic resistance in most environmental bacteria is due to the acquisition of new genes, often associated with the mobile elements (Jiang and Paul 1998).

Hospital wastewater treatment plants have been found to be an important reservoir for antibiotic resistant bacteria and resistance plasmids (Kummerer 2001; Brown et al., 2006; Szczepanowski et al., 2009; Diwan et al., 2010; Chagas et al., 2011; Diwan et al., 2012). Antibiotic resistance gene can enter into aquatic environments by discharging of the effluent and sludge to the environments (Auerbach, et al., 2007) and can transfer to the endogenous microbial community.

Among the high prevalence of beta-lactam resistant bacteria in the hospital and community acquired infection reported in Thailand (Danchaivijitr et al., 2007; Kiratisin, Apisarnthanarak et al. 2007; Apisarnthanarak et al., 2008; Niumsup et al., 2008; Boonkerd et al., 2009), CTX-M, TEM and SHV-type beta-lactamase are emerging resistance determinants in Enterobacteriaceae. During December 2004 to May 2005, the prevalence of bla_{CTX-M}, bla_{TEM} and bla_{SHV} were strikingly high in *Escherichia coli* (99.6%, 77.0%, 3.8%) and *Klebsiella pneumonia* (99.2%, 71.7%, 87.4%) (Kiratisin *et al*., 2008).

2. Objective

To detect the CTX-M, TEM and SHV-type beta-lactamase genes in hospital wastewater effluent.

3. Methodology

3.1 Final effluent sample collection

One liter of surface water was taken from final effluent tank of a hospital wastewater treatment plant. Water sample was aseptically collected, 15 centimeters depth from surface water, in a sterile bottle. The effluent water was filtrated through a 0.8 μm membrane filter (Nalgene, USA) to remove large contaminated particles and bacterial cells harvested on 0.1 μm membrane filter (Nalgene, USA), using vacuum pressure of 15 to 20 lb/in^2. The membrane was removed from the filter apparatus, cut into small pieces, placed along the side of 50 ml sterile conical tube and soaked with 10 ml sterile TE buffer. Bacteria were harvested by centrifugation at 11,000 x *g* for 1 minute. Supernatant was discarded and the pellet was used for metagenomics plasmid library construction.

3.2 Metagenomics plasmid library construction

Metagenomics plasmids were extracted from bacterial pellet using plasmid DNA purification kit (Macherey-Nagel, Germany) according to manufacturer's instructions. Briefly, bacterial cell pellet was resuspended in lysis buffer, alkaline denaturation, neutralization and binding to silica membrane column. After washing, the purified plasmids were collected and submitted to the ethidium bromide containing agarose gel electrophoresis and visualized by Gel Documentation system (BIS 303 PC, DNR Bio-Imaging Systems, Israel) to estimate the plasmid concentration. The purified plasmids were electroporated to *E. coli* DH5α to construct a metagenomics DNA library using micropulser (Bio-Rad, USA). The library was kept at -80°C until used for screening of the beta-lactam resistant determinants.

3.3 Screening for beta-lactam resistant clones

The library was thawed rapidly, centrifuged, and the pellet was resuspended in 5 ml of LB broth containing ampicillin at the final concentration of 50 μg/ml and incubated at 37 °C in a shaking incubator (200 rpm) overnight. The overnight culture was centrifuged, and the pellet was resuspended in 5 ml of fresh LB broth containing ampicillin at the final concentration of 100 μg/ml and incubated at 37 °C in a shaking incubator (200 rpm) overnight. This step was repeated until bacteria grew well in LB broth containing ampicillin at the final concentration of 100 μg/ml. This ampicillin resistant culture was aliquot and stored at – 80 °C.

3.4 Detection of bla_{CTX}, bla_{TEM} and bla_{SHV} beta-lactamase genes

Thirty ampicillin resistant clones were selected for detection of bla_{CTX}, bla_{TEM} and bla_{SHV} beta-lactamase genes. One colony from each of the selected clones was cultivated in 20 ml LB broth containing 100 μg/ml ampicillin, and incubated with shaking at 37 °C for 24 hours. Bacterial cells were harvested by centrifugation at 11,000 x *g* for 1 minute. Their plasmids were extracted using plasmid DNA purification kit (Macherey-Nagel, Germany) as described. Purified plasmid were stored at -20 °C until being used in PCR amplification.

Oligonucleotide primers were designed from the consensus sequences and summarized in Table 1. Primers CTX-M, TEM, and SHV were used to identify $bla_{CTX\text{-}M}$, bla_{TEM}, and bla_{SHV}, respectively. PCR was carried out in a thermal cycler (MJ Mini Gradient Thermal cycler, Bio-Rad, USA) using 0.2 ml PCR tubes. The reaction mixture composed of 1X ThermoPol reaction buffer (200 mM Tris-HCl pH 8.8, 100 mM $(NH_4)_2SO_4$, 100 mM KCl, 20 mM Mg_2SO_4, 1.0% Triton X-100; New England BioLabs, USA), 200 μM of each deoxynucleoside triphosphate (dNTPs), 250 nM of each primer, 1 unit of *Taq* DNA polymerase (New England BioLabs, USA), approximately 10 ng of plasmid DNA template including positive control, and Milli-Q water to a final volume of 20 μl. The mixture were amplified by using the following steps; an initial denaturation at 95 °C for 2 minutes and followed by 35 cycles of denaturation at 95 °C for 15 seconds, annealing at 53-

55 °C for 30 seconds, and extension at 68 °C for 30 seconds, and a final extension at 68 °C for 5 minutes. PCR products were read by gel documentation system and were interpreted for beta-lactam resistance genes.

Table 1 List of primers used for PCR amplification of beta-lactam resistance genes

Primer Name	Target	Accession number	Sequence (5'to 3')	Position	Annealing Temp (°C)	PCR product (bp)
CTX-M-F	*bla* CTX	X92506	GCA GYA CCA GYA ARG TKA TGG C	232 - 253	55	511
CTX-M-R	*bla* CTX		CDC MGC TGC CGG TYT TAT CVC	724-704		
TEM-F	*bla* TEM	DQ909059	TCG GAG GAC CGA AGG AGC	419 - 436	54	228
TEM-R	*bla* TEM		CTG CAA CTT TAT CCG CCT CC	646 - 627		
SHV-F	*bla* SHV	EF373975	AGC GAA AGC CAG CTG TCG	94 -111	53	682
SHV-R	*bla* SHV		TAT YCG GGC YAA GCA GGG C	745 - 727		

Y = C or T; R = A or G; K = G or T; D = A or G or T; M = A or C; V = A or C or G

4. Results

4.1 Metagenomics plasmid library

Metagenomics DNAs were extracted from total bacterial cells contained in 1 liter of the final effluent. A total of 50 µl of purified plasmid DNAs were obtained and used to construct a metagenomics DNA library, by electroporation to *E. coli* DH5α competent cells. Totally 10 electroporation had been done. The library was expanded and used for screening of beta-lactam antibiotic resistant clones.

4.2 Beta-lactamase producing clones

Aliquots of the metagenomics plasmid library were screened for ampicillin resistant clones by growing in LB broth containing ampicillin (100 µg/ml). In order to avoid the redundant clones, merely 3 colonies per transformation were selected for characterization. The average plasmid sizes were >20 kb (Fig. 1).

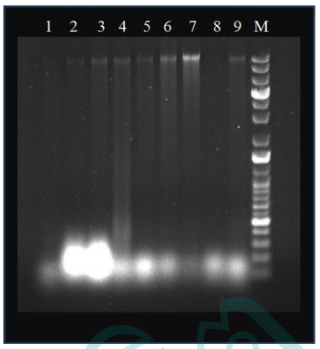

Fig. 1 Agarose gel electrophoresis of plasmid extracted from clone 1-9 (Lane 1-9). Lane M:GeneRuler 1 kb DNA Plus Ladder (Fermentuas, Inc., MD, USA)

4.3 Detection of bla_{CTX}, bla_{TEM} and bla_{SHV} beta-lactamase

The results showed that bla_{TEM} were identified in all of the 30 clones, whereas none of them positive for bla_{CTX-M}, and bla_{SHV}. Gel electrophoresis of the beta-lactamase genes amplicons are shown in Figure 2. Nucleotide sequence analysis of the PCR products were performed on both strands. Similarity searching was performed using online BLAST (Basic Local Alignment Search Tool).

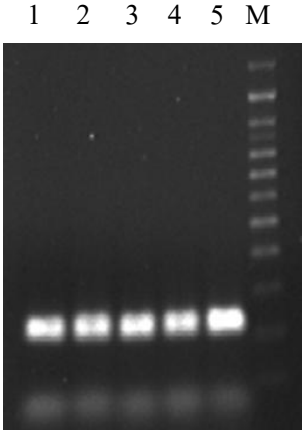

Fig. 2 Agarose gel electrophoresis of bla_{TEM} PCR products (Lane 1-4). Lane 5 positive control, Lane M:GeneRuler 1 kb DNA Plus Ladder (Fermentuas, Inc., MD, USA) Lane M: 100 bp DNA ladder (New England BioLabs, USA)

5. Discussion

Antibiotics are empirically used in hospitals. They are extracted into the urine and discharged into hospital wastewater (Lindberg et al., 2004), an important source of both antibiotic resistant bacteria and antibiotic residues (Watkinson et al., 2007). Wastewater treatment plants (WWTPs) use a variety of microorganisms in the treatment processes. Once antibiotic resistant bacteria reach WWTPs they potentially can disseminate their resistance genes among members of the endogenous microbial community (Tchobanoglous et al., 2003). There are several reports stated that WWTPs are reservoirs for diverse antibiotic resistance elements (Tchobanoglous et al., 2003; Parsley, Consuegra et al., 2010; Merlin et al., 2011). The presence of low concentration of antibiotics in WWTPs may exert selective pressure favoring resistant strains. Incomplete elimination of bacteria and antibiotics during wastewater treatment results in the entry of antibiotics and antibiotic-resistant bacteria into natural environment (Dolejska et al., 2011). These brought increasing concerns regarding their contribution to the presence and persistence of resistant pathogenic and non-pathogenic bacteria in the natural water environment. Due to the fact that transformation efficiencies obtained by calcium chloride treatment are 10^6 to 10^7 (Mandel, 1970) whereas the electroporation method are greater than 10^9 transformants (Calvin, 1988), in the present study, plasmid DNAs were extracted from bacteria contained in one liter of a final effluentfrom a hospital WWTP and electroporated to *E. coli* DH5α. In the present study, plasmid DNAs were extracted from bacteria contained in one liter of a final effluent from a hospital WWTP and electroporated to *E. coli* DH5α. The metagenomics plasmid library was screened for beta-lactam resistance and 30 clones were selected for genotypic characterization.

The plasmids with average sizes >20 kb were evaluated for beta-lactam resistance genes. In the present study, bla_{TEM} was the most predominant beta-lactamase encoding genes (100%). TEM-type beta-lactamase (class A) are most often found in Gram negative bacteria such as *E. coli* and *K. pneumoniae* (Al-Jassser, 2006). More than 200 TEM-type beta-lactamases have been described and majority of which are extended spectrum beta-lactamase (ESBLs). TEM-type ESBL is responsible to hydrolyze third-generation cephalosporin, and it is resistant to inhibitor. bla_{TEM} can be easily spread by horizontal transfer especially when they located on broad host range plasmids (Gotz et al., 1996). This enzyme group has been known to play an important role in human infection worldwide (Bradford 2001; Blanco et al., 2009; Kanamori, Yano et al., 2012), with no exception for Thailand (Apisarnthanarak et al., 2008; Kiratisin et al., 2008; Pongpech et al., 2008; Sasaki, H et al., 2010). The enzyme group has been detected in hospital wastewater from many countries such as India (Diwan et al., 2012), Brazil (Chagas et al., 2011), and Czech Republic (Dolejska et al., 2011). To the best of our knowledge, this is the first report on the presence of bla_{TEM} genes in hospital wastewater in Bangkok, Thailand.

This study not only revealed the leakage of bacteria carrying antibiotic resistant determinants from a good practice WWTP, but also exhibited a possible route of antibiotic resistance dissemination from hospital to community. Hopefully, this finding could bring public concerns about the dissemination of antibiotic resistance determinants.

6. Conclusions

In the present study, a metagenomics plasmid library was constructed and thirty beta-lactam resistant clones were characterized. The antibiotic resistant genes, namely bla_{TEM} was found in all of the tested clones. The data clearly showed that bacteria harboring antibiotic resistant plasmids have evaded from the treatment processes. These findings indicated that hospital wastewater treatment plants have a potential to disseminate antibiotic resistant determinants to the environmental bacteria.

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