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Resistance Genes in Multiply-resistant *Pseudomonas aeruginosa* Clinical Isolates from Siriraj Hospital

Chanwit Tribuddharat, M.D., Ph.D.*
Michael A. Fennewald, Ph.D.**

Abstract : **The *P. aeruginosa* clinical isolates with multiple antibiotic resistance from Siriraj Hospital showed a region of closely related integron elements and transposons which carried at least 10 antibiotic resistance genes. The resistance genes from PaTh2 were directly cloned and the genes were sequenced within the total length of 26 kb. Resistance to aminoglycosides was conferred by three different aminoglycoside transferase genes, *aadA2*, *aph*, and *aadB*. Resistance to β-lactams was conferred by three different β-lactamases, bla_{PSE-1}, bla_{CEF-1} (also called bla_{VEB-1}), and bla_{OXA-10}. Rifampin resistance was conferred by the *arr-2* gene which encoded for a ribosylating transferase. Resistance to chloramphenicol was conferred by an active transport protein encoded by the *cmlA* gene. The resistance genes to quaternary ammonium compound and sulfonamide were also identified, *qacEΔ1* and *sul1*, respectively. The *arr-2* gene was further analyzed to confirm rifampin resistance in laboratory strains of *E. coli* and *P. aeruginosa*. The CEF-1 β-lactamase was purified for kinetic studies. The oxy-imino cephalosporins were good substrates for CEF-1 β-lactamase, whereas ampicillin was the poorest substrate. Most antibiotic resistance genes were located on gene cassettes as parts of two integron elements, and some were not cassettes but were connected to this region by unknown mechanism of recombination. The adjacent transposon genes indicated the possibility of spread of this large collection of antibiotic resistance genes among other bacteria as seen in 4 other clinical isolates of *P. aeruginosa*. The sequence adjacent to the resistance cluster showed no homology to any sequence in the GenBank implying the cluster was not chromosomal in origin.**

**Department of Microbiology, Faculty of Medicine Siriraj Hospital, Mahidol University, Thailand, **Department of Microbiology and Immunology, Finch University of Health Sciences/The Chicago Medical School, Illinois, U.S.A.*

เรื่องย่อ : ยีนดื้อยาของเชื้อ *Pseudomonas aeruginosa* ที่ดื้อต่อยาหลายขนานที่แยกได้ในโรงพยาบาลศิริราช

ชาญวิทย์ ตรีพุทธรัตน์ พ.บ., Ph.D.*, Michael A. Fennewald, Ph.D.**

**ภาควิชาจุลชีววิทยา, คณะแพทยศาสตร์ศิริราชพยาบาล, มหาวิทยาลัยมหิดล, กรุงเทพมหานคร,*
***Department of Microbiology and Immunology, Finch University of Health Sciences/ The Chicago Medical School, Illinois, U.S.A.*

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เชื้อ *Pseudomonas aeruginosa* ที่แยกได้จากผู้ป่วยที่มารับการรักษาที่ ร.พ.ศิริราช เป็นเชื้อที่ดื้อต่อยาต้านจุลชีพหลายชนิด จากการศึกษาในครั้งนี้พบว่าสายพันธุ์ PaTh2 มียีนควบคุมการดื้อยาที่อยู่เรียงต่อกันเป็นชุดอย่างน้อย 10 ยีน การศึกษายีนที่ควบคุมการดื้อยาทั้ง 10 ชนิดทำโดยนำยีนไปไว้ในเชื้อสายพันธุ์ที่ใช้ในห้องปฏิบัติการซึ่งไม่ดื้อยาให้กลายเป็นสายพันธุ์ที่ดื้อยาหลายชนิดได้ เช่นเดียวกับเชื้อ PaTh2 กลุ่มยีนที่เป็นเหตุให้เชื้อดื้อยา ได้ถูกนำมาวิเคราะห์หาลำดับเบส พบว่ามีความยาวประมาณ 26 kb (~26,000 bases) การดื้อยาในกลุ่ม aminoglycosides พบว่าเกิดจากยีน 3 ชนิดที่สร้างเอนไซม์ transferase ได้แก่ *aadA2*, *aph* และ *aadB* การดื้อยาในกลุ่ม β-lactam เกิดจากการสร้างเอนไซม์ β-lactamase สามชนิดโดยยีน bla_{PSE-1}, bla_{CEF-1} (มีอีกชื่อว่า bla_{VEB-1}), และ bla_{OXA-10} การดื้อยา rifampin เกิดจากยีน *arr-2* ที่สร้างเอนไซม์ ribosylating transferase ส่วนการดื้อยา chloramphenicol เกิดจากการขับยาออกจากเซลล์โดยการทำงานของ efflux pump protein ซึ่งสร้างโดยยีน *cmlA* นอกจากนี้ยังพบว่ามียีน *qacEΔ1* และ *sul1* ซึ่งทำให้เชื้อดื้อต่อ antiseptic และ sulfonamide ยีน *arr-2* ได้ถูกนำมาศึกษาต่อเพื่อยืนยันถึงความสามารถในการทำให้เชื้อดื้อยา rifampin ในเชื้อ *E. coli* และ *P. aeruginosa* ส่วนเอนไซม์ CEF-1 β-lactamase ได้ถูกนำมาสังเคราะห์และใช้ในการศึกษาคุณสมบัติของเอนไซม์ พบว่า oxy-imino cephalosporins เป็น substrate ที่ดีสำหรับเอนไซม์แต่ ampicillin เป็น substrate ที่ไม่ดี ยีนควบคุมการดื้อยาที่พบส่วนใหญ่มีลักษณะที่เรียกว่า gene cassette ซึ่งเป็นส่วนหนึ่งของ integron element จำนวนสองชุด ส่วนยีนดื้อยาบางยีนพบว่ามาเรียงต่อใกล้ ๆ กันโดยกระบวนการ recombination ที่ไม่ทราบกลไกแน่ชัด อย่างไรก็ตามยีนนั้นไม่มีคุณสมบัติของ gene cassette ของ integron element นอกจากนี้ยังพบว่ามียีนของ transposon ที่อยู่ถัดออกไป ซึ่งบ่งว่าความสามารถในการแพร่กระจายของยีนควบคุมการดื้อยาเหล่านี้อาจเกิดขึ้นได้โดยง่าย ยีนข้างเคียงที่อยู่ถัดออกไปอีกเป็นยีนที่ไม่เหมือนยีนใด ๆ ใน GenBank database ซึ่งน่าจะเป็นไปได้ว่ายีนเหล่านี้ไม่ใช่ยีนบน chromosome ของเชื้อ แต่น่าจะเป็นยีนที่ได้รับการถ่ายทอดมาจากเชื้ออื่น

INTRODUCTION

Pseudomonas aeruginosa is a non-fermentative Gram negative rod that can be found in water, soil, fresh fruit and vegetables, and sometimes in the human gastrointestinal tract. It is distributed worldwide and has become a major problem as one of the common pathogens isolated in hospitals, and as a pathogen showing multiple antibiotic resistance. Even though it seems to have low virulence, the mortality rate among those who get infected is very high, in some reports, up to 70%[1]. *P. aeruginosa* is a very versatile organism, it can survive in various harsh conditions, such as in heavy metal contaminated water, on soap, and even in some antiseptics[2]. One of

the key successes for this organism is the ability to acquire useful genes from other organisms that can make them survive in many types of selective pressure. The phenomenon of selective pressure can also be seen in the hospital situation, where many antibiotics have been used to treat patients. The strains that have been isolated from hospitals or nosocomial strains always show a higher level of resistance to many antibiotics than those isolated from patients with community-acquired infections. In general, most bacteria become antibiotic resistant by certain mechanisms, for examples, modification of the targets of antibiotics, inactivation of antibiotics, or a decrease in the concentration of antibiotics inside the cells, etc[1]. The selection of mechanisms may be unique and depend on both the bacterial species and the antibiotic class in use. *P. aeruginosa* is intrinsically resistant to many antibiotics, and there is evidence showing that *P. aeruginosa* can share multiple antibiotic resistant plasmids with other enteric bacteria[4,5]. Although, there have been reports from some countries that use a lot of antibiotics, such as South America and some European countries, about the mechanisms used by multiply resistant *P. aeruginosa*, there are not many studies that identify the genetic background of this organism in Thailand[6,7]. In this report we show the different mechanisms selected by this organism to produce multiple antibiotic resistance. We also show that the resistance genes are collectively acquired by the mobile genetic element called the "integron element"[8-11]. There are also other elements, such as insertion sequence elements and transposons that play a further role in collecting the antibiotic resistance genes and forming a more complicated island of resistance genes. Of special note, the different types of resistance genes found in this study were both directly and indirectly involved with the quantity and frequency of antibiotic use in the treatment of pseudomonal infection at Siriraj Hospital.

MATERIALS AND METHODS

Enzymes and chemicals

The restriction enzymes, T4 DNA ligase, calf intestinal phosphatase, and Klenow fragment of DNA polymerase I were purchased from either Promega or New England Biolabs, and were used according to the company's instructions for their own optimum conditions. Sarkosyl was purchased from CIBA-GEIGY. The isotope [α-^{32}p]dCTP was purchased from Amersham.

Bacterial strains and plasmids

Strains and plasmids used in the study are listed in Table 1.

β-lactamase assay with cell-free extract

The β-lactamase assay was performed by using the cell-free extract from *E. coli* carrying the plasmid construct that contains bla_{PSE-1} or bla_{CEF-1} or no plasmid. The β-lactamase was recovered from cells subjected to a freeze-thaw procedure. The 500-ml saturated culture was centrifuged and washed with 0.1 M potassium phosphate buffer (pH 7.0), and resuspended in 8 ml of 0.1 M potassium phosphate buffer (pH 7.0), and subjected to a freeze-thaw procedure. The cell suspension was frozen in liquid nitrogen for 5 min and thawed in ice-cold water for 1 h (three cycles). The cell debris was centrifuged at 0°C, 100,000g in a Beckman L-70 ultracentrifuge machine for 30 min to obtain a cell-free extract. The hydrolysis of carbenicillin or ceftazidime was determined by spectrophotometer readings at 210 nm or 256 nm, respectively.

DNA preparation and analysis

Plasmid DNA was prepared from *E. coli* or *P. aeruginosa* by the alkaline-lysis method[15]. Total DNA was prepared from *P. aeruginosa* PaTh2 as described by Strom and Lory[13]. Briefly, an over-night culture of *P. aeruginosa* was washed twice with 10 mM Tris (pH 8.0), then resuspended in 10 mM Tris (pH 8.0)-10 mM EDTA containing 1 mg/ml of lysozyme. The cells were incubated for 15 min at 37°C, and were lysed by the addition of Sarkosyl and proteinase K to the final concentration of 1% and 50 μg/ml, respectively. The lysate was incubated for an additional 30 min at 37°C, followed by three phenol and two chloroform extractions. The DNA was then precipitated using ethanol, spooled out, and resuspended in TE (10 mM Tris, 1 mM EDTA [pH 8.0]).

DNA cloning of antibiotic resistance genes

P. aeruginosa PaTh2 DNA (100 μg) was digested over night with *Eco*RI, *Bam*HI, or *Xho*I

Table 1. Strains and plasmids.

Strain or plasmid	Relevant characteristics	Source or reference
Strains		
P. aeruginosa		
PaTh1-5	multidrug resistant clinical isolates	From Siriraj Hospital, Mahidol University, Thailand
PAC5	laboratory strain, ceftazidime sensitive	P.H. Clarke[12]
PAO1	laboratory strain, ceftazidime sensitive	P.H. Clarke[12]
PAK	laboratory strain, ceftazidime sensitive	Stephen Lory[13]
E. coli		
XL1-Blue	*recA1*, *Tet*^{r}	Stratagene
DH5αF'	*recA1*, *Nal*^{r}	Stratagene
Plasmids		
pUCP24	*E. coli-Ps. aeruginosa* shuttle vector with gentamicin resistant gene, *aacC1*	H.P. Schweizer[14]
pBluescript SK(+)	*ColE1* cloning vector with ampicillin resistant gene, bla_{TEM-1}	Stratagene
pCTF101	pUB5572 with *Carb*^{r} transposon	This study
pCTF102	11-kb *Eco*RI fragment from PaTh2 inserted into pUCP24	This study
pCTF104	4.2-kb *Bam*HI fragment from PaTh2 inserted into pUCP24	This study
pCTF202	13-kb *Xho*I fragment from PaTh2 inserted into pBluescript SK(+)	This study

restriction enzyme, and the reactions were stopped by heating at 65°C for 30 min. The digested DNA was then ligated with pUCP24 cut with the relevant enzyme. The ligation with T4 DNA ligase was carried out at 30°C over night at vector-to-insert ratios of 1:5. The ligation mixture was used to transform the competent *E. coli* directly, and the transformants were selected on LB agar containing 100 µg/ml of ampicillin or 100 µg/ml of ceftazidime.

Southern blot analysis

The *Eco*RI and/or *Bam*HI digested total DNA from *Pseudomonas* and plasmids were electrophoresed in 1% agarose gel, and were then transferred to a positively-charged nylon membrane in alkaline buffer (0.4 N NaCl). The DNA probes were prepared from plasmid pCTF102 or pCTF104, and labeled with [α-^{32}p]dCTP by random priming (multiprime DNA labelling system, Amersham). The nylon membranes were prehybri-dized at 42°C for 24 h in 20 ml of 50 % formamide, 5X Denhardt's solution, 1 M NaCl, 0.1 M Tris, 1 mM EDTA, 0.05 M sodium phosphate pH 6.8. The denatured probe was later added to the mixture and the membrane was hybridized under stringent conditions.

Purification of β-lactamase and the kinetic analysis

Purified β-lactamase was used to study the kinetic parameters of the enzyme. *E. coli* carrying pCTF104 was used to obtain the CEF-1 β-lactamase enzymes. The cells were grown in 3 liters of LB media containing 50 µg/ml of ceftazidime. After growing the cells to the late exponential phase, the cells were harvested and the periplasmic fraction including the β-lactamase was obtained by an osmotic shock procedure. The DEAE-sephacel and CM-Sepharose CL-6B ion-exchanger columns were used for purification. The fraction containing β-lactamase was determined by nitrocefin hydrolysis and SDS-PAGE. The Δε (the extinction coefficient), V_{max}, K_m, and k_{cat} were determined using the following

equations. $\Delta\varepsilon = \frac{OD_S - OD_P}{L \times [S]}$, $V = \frac{\Delta OD}{L \times \Delta\varepsilon \times 60}$, $k_{cat} = \frac{V_{max}}{[E]}$, respectively[16-18]. Then the values from different β-lactams were compared.

DNA sequencing and gene comparison

The DNA sequences were obtained by using a 377XL automated DNA sequencer operated by the University of Chicago Cancer Research Center. The results were then used as inputs to search for homologous sequences in GenBank with the local alignment algorithm BLASTN or BLASTX. For the ceftazidime and rifampin resistance cassettes, the genes were translated to proteins and then the protein sequences were used as inputs for the ClustalW program to align with other protein homologs.

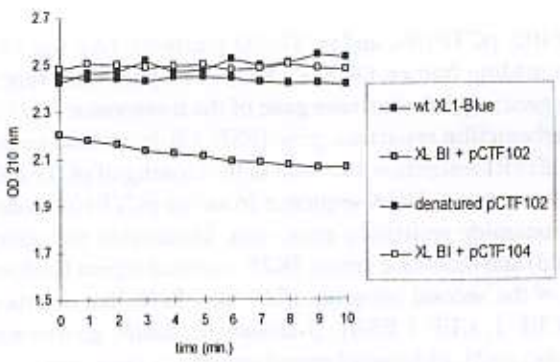
Nucleotide sequence and accession number

The gene sequence was accessed from the thesis by Tribuddharat, C. and part of the gene sequence was also accessed from the GenBank database (accession number AF078527)[19].

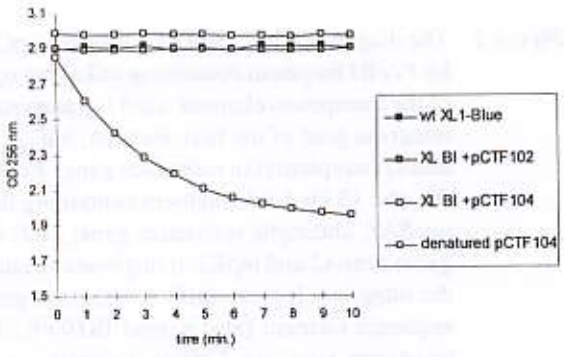
RESULTS

β-lactamase assay with cell-free extract

The cell-free extract obtained from *E. coli* carrying pCTF102 and pCTF104 plasmids that contained *bla*PSE-1 and *bla*CEF-1 showed the ability of the enzymes to hydrolyze carbenicillin and ceftazidime, respectively. The heat-treated extracts showed no enzymatic activity indicating that the protein is responsible for hydrolysis. (Figure 1)



(A) carbenicillin as substrate

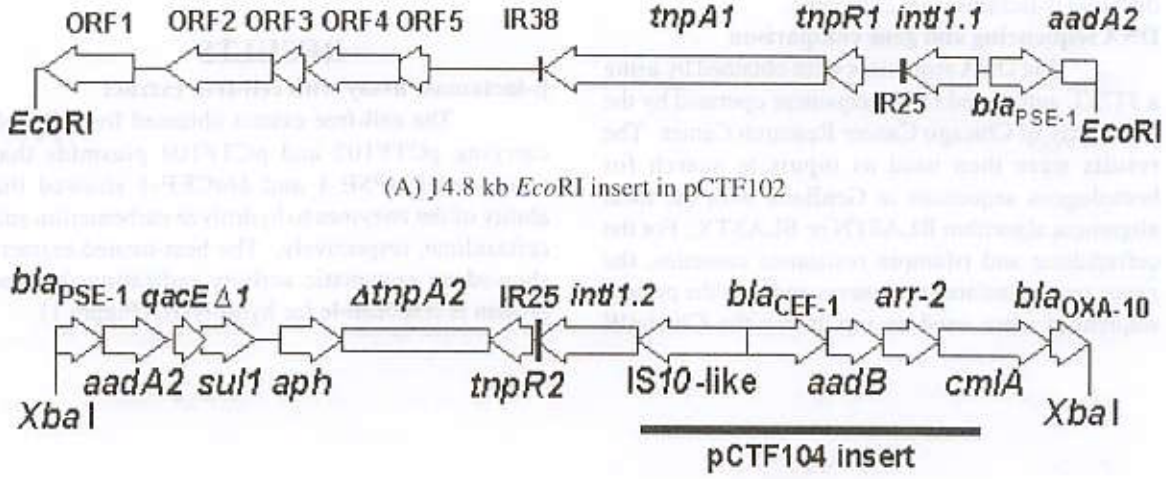


(B) ceftazidime as substrate

Figure 1. The hydrolysis of carbenicillin (A) and ceftazidime (B) using crude extracts from XL1-Blue *E. coli* carrying β-lactamase clone from PaTh2. OD 210 nm = the wavelength used for carbenicillin in UV spectrophotometry, OD 256 nm = the wavelength used for ceftazidime in UV spectrophotometry. Denatured extracts have been treated at 60°C for 1 hour.

DNA cloning of antibiotic resistance genes

The *Eco*RI- and *Bam*HI-digested DNA from PaTh2 gave two clones of plasmids, pCTF102 and pCTF104, which made *E.coli* resistant to carbenicillin and ceftazidime, respectively. We cloned pCTF202 using *Xba*I restriction enzyme to get the continuation of the fragments from pCTF102 and pCTF104 showing that the resistance genes originated from a large cluster of antibiotic resistance genes. (Figure 2)



(A) 14.8 kb *Eco*RI insert in pCTF102

(B) 13 kb *Xba*I insert in pCTF202

Figure 2. The diagrams show the DNA inserts in pCTF102, pCTF104, and pCTF202 plasmids. (A), the 14.8 kb *Eco*RI fragment containing unknown open reading frames, ORF1-5; IR38, 38-bp inverted repeat of the transposon element; *tnpA1*, transposase gene; *tnpR1*, resolvase gene of the transposon; *intI1.1*, integrase gene of the first integron; bla_{PSE-1}, carbenicillin resistance gene (PSE-1 β-lactamase gene); *aadA2*, streptomycin resistance gene; *Eco*RI, *Eco*RI restriction site used in the cloning of pCTF102. (B), the 13 kb *Xba*I fragment containing the downstream DNA sequence from the pCTF102 insert; *qacEΔ1*, antiseptic resistance gene; *sul1*, sulfonamide resistance gene; *aph*, kanamycin resistance gene; Δ*tnpA2* and *tnpR2*, transposase (truncated) and resolvase genes; IR25, inverted repeat flanking the integron element; *intI1.2*, integrase gene of the second integron; IS*10*-like, IS*10*-like insertion sequence element (also named IS*1999*); *bla*CEF-1, CEF-1 ESBL β-lactamase; *aadB*, gentamicin resistance gene; *arr-2*, rifampin resistance gene; *cmlA*, chloramphenicol resistance gene; bla_{OXA-10}, oxacillin resistance gene; *Xba*I, restriction sites used in the cloning of pCTF202.

Southern blot analysis

The Southern blot analysis was used in order to confirm that the source of resistance genes was from PaTh2. The radioactively-labeled pCTF102 DNA clearly showed the presence of the inserted fragment in PaTh2. Clones and other clinical isolates also showed intensely hybridized bands indicating the existence of common DNA fragments among them. There was no cross-hybridization to laboratory strains with the pCTF102 probe. The results of the Southern blot analysis using pCTF104 as a radioactive probe also showed similar hybridization

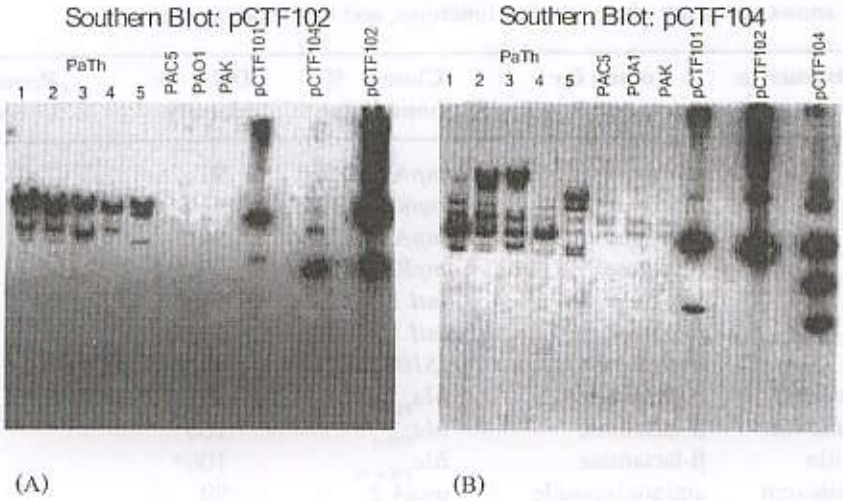


Figure 3. Southern blot hybridization. (A), total DNA from PaTh1-5 and plasmid clones digested with *Eco*RI were used as targets for the ^{32}P-radioactive-labeled pCTF102 probe. (B), total DNA from PaTh1-5 and plasmid clones digested with BamHI were used as targets for the ^{32}P-radioactive-labeled pCTF104 probe. Plasmids were positive controls, and PAC5, PAO1, and PAK that were laboratory strains were negative controls.

among clinical isolates. However, there were faint hybridized bands from laboratory *P. aeruginosa* isolates. The explanation may be that the insertion sequence element found in pCTF104 clone can find relatively homologous insertion sequence elements in laboratory strains.

DNA sequencing and gene comparison

The results of DNA sequencing revealed a large cluster of antibiotic resistance genes. Table 2 shows the list of antibiotic genes including genes involved in transposition found in transposons in the PaTh2 strain.

Purification of β-lactamase and the kinetic analysis

The CEF-1 β-lactamase was purified using two different ionic exchanger columns, and the purity of the enzyme was more than 90% (data not shown). The kinetic study revealed that CEF-1 had a very good hydrotic activity against cephalosporins including third generation ones, so the enzyme might be categorized as a cepharosporinase and could be identified as the extended-spectrum β-lactamase (ESBL). According to the amino acid sequence of CEF-1, it belongs to class A β-lactamase, and it is susceptible to clavulanic acid inhibition (data not shown). For kinetic parameters, the *Km* may infer interaction of the enzyme and substrate with lower values indicating better affinity. The k_{cat} indicates the rate of hydrolysis of the substrate. Therefore, the k_{cat}/K_m would be a better parameter to indicate the relationship between the enzyme and different substrates, the higher the value, the better the hydrolysis of that substrate by the enzyme. (Table 3)

DISCUSSION

It is a well-known fact that bacterial strains isolated from hospital environment show greater resistance to antibiotic (than strains isolated from community), although in most circumstances health care personnels seem to ignore the cause of this serious problem. Studies that aim to address this issue in a developing country like Thailand are scarce and it is difficult to obtain useful data, due to the large number of bacterial species and strains and varieties with resistant phenotypes. However, there should be data available from each geographic area or from each hospital so that the physicians and other health care personnels can be aware of the seriousness of the antibiotic resistance problem and can monitor the resistance gene pool in their own hospitals.

Table 2. The list shows the genes, their putative functions, and their homologues.

Name	Resistance to	Coding for	Closest % homologue	DNA % identity	Protein identity/ similarity
tnpA 1	-	transposase	*tnp*A , Tn*501*	91	96/97
tnpR 1	-	resolvase	*tnp*R , Tn*501*	91	92/95
tnpA 2	-	truncated transposase	*tnp*A , Tn*3926*	99*	99*
tnpR 2	-	resolvase	*tnp*R , Tn*3926*	99	99/99
intI 1.1	-	integrase	*intI* 1 , Tn*21*	99	100/100
intI 1.2	-	integrase	*intI* 1 , Tn*21*	99	100/100
IS10-like	-	transposase	*IS10R*, Tn*10*	48	68/83
bla_{CEF-1}	ceftazidime	β-lactamase	bla_{PER-1}	53	38/62
bla_{PSE-1}	carbenicillin	β-lactamase	bla_{PSE-1}	100	100/100
bla_{OXA-10}	oxacillin	β-lactamase	bla_{OXA-10}	100*	100*
aadA 2	streptomycin	aminoglycoside adenyltransferase	*aadA 2*	99	99/100
qacE Δ *1*	antiseptics	ethidium bromide resistance protein	*qacE* Δ *1*	100	100/100
sul 1	sulfonamide	dihydropteroate synthase	*sul 1*	100	100/100
aph	kanamycin	aminoglycoside phosphotransferase	*aph*	100	100/100
aadB	gentamicin	aminoglycoside adenyltransferase	*aadB*	100	100/100
arr- 2	rifampin	rifampin ribosyltransferase	*arr*	57	54/68
cmlA	chloramphenicol	chloramphenicol transporter	*cmlA*	99	98/99

* = incomplete sequence.

Table 3. Enzyme kinetic parameters obtained from different β-lactam antibiotics. Units for *Vmax, Km, kcat,* and *kcat/ Km* are nm. S^{-1} , μM, S^{-1}, and $mM^{-1}.S^{-1}$ respectively.

Substrate	V_{max}	K_m	k_{cat}	k_{cat}/K_m	Relative k_{cat}/K_m
Ampicillin	0.411 ± 0.02	378 ± 48.95	5.3 ± 0.25	14	100
Cephaloridine	0.047 ± 0.01	32 ± 16.59	15.8 ± 3.05	492	3,490
Cefazolin	0.100 ± 0.01	22 ± 3.43	16.6 ± 0.92	764	5,418
Cefuroxime	0.140 ± 0.01	50 ± 6.94	31.1 ± 2.19	624	4,428
Ceftriaxone	0.048 ± 0.003	131 ± 25.74	6.4 ± 0.43	49	346
Ceftazidime	0.136 ± 0.02	265 ± 83.49	176.3 ± 22.85	666	4,719
Aztreonam	0.686 ± 0.14	48 ± 23.90	11.4 ± 2.37	238	1,688

Physicians can therefore learn what antibiotics are no longer usable and what antibiotics will promote resistance and cross-resistance either directly or indirectly. This study was aimed firstly to address the situation at Siriraj Hospital, and to give useful data about how serious the problem was. We selected *P. aeruginosa*, because it was one of the most antibiotic resistant species and had a high prevalence. We found that the resistant strains had a similar genetic element named the "integron element". The features of this element include its mobility, enhancing its spread among different species, its ability to collect any antibiotic/antiseptic resistance genes, and its flexibility to adapt or adjust the expression of resistance genes according to bacterial needs[20]. This study has identified at least ten antibiotic/antiseptic resistance genes from a single strain of *P. aeruginosa* PaTh2. There were three different aminoglycoside transferase genes, *aadA2*, *aph*, and *aadB*, each of which could produce an enzyme that could modify streptomycin, kanamycin, and gentamicin, respectively, so they could no longer bind to their own targets, the ribosomal subunits. There were also three different β-lactamase genes, bla_{PSE-1}, bla_{CEF-1} (also called bla_{VEB-1}), and bla_{OXA-10} which could make bacteria become highly resistant to carbenicillin, extended-spectrum cephalosporins and monobactam, and oxacillin, respectively[19,21,22]. Although, these β-lactamases seem to have their own preferred substrates, they theoretically should be able to exert a synergistically hydrolytic property towards poor substrate, such as carbapenems. If we consider the chromosomal AmpC type β-lactamase (Ambler class C[23,24]) of *P. aeruginosa*, there are four different β-lactamases this single strain can use in the β-lactam antibiotic treatment battle. PaTh2 also carried the resistance gene for rifampin using a novel method identified in *P. aeruginosa* in which rifampin was modified by ADP-ribose attaching to its target-binding site[21]. Chloramphenicol resistance was produced by an efflux mechanism using CmlA protein[25,26]. The CmlA efflux might also show low level tetracycline resistance. Antiseptic resistance by *qacEΔ1* was also mediated by the efflux mechanism[27]. Sulfonamide resistance was conferred by a by-pass mechanism using a new enzyme encoded by the *sul1* gene[28]. Not only does the PaTh2 strain carry resistance genes against commonly used antibiotics, but it also carries resistance genes for antibiotics that are apparently not used for pseudomonal infection or are no longer used, such as rifampin and kanamycin, etc. This finding indicates the fact that the resistant strains have no trouble carrying many antibiotic resistance genes simultaneously, and antibiotic cycling may not be a very effective approach. From the gene sequence data, the ORF1-5, sequence upstream to the antibiotic resistance gene cluster found no homologue in the GenBank database, indicating that the resistance gene cluster was on the very large plasmid, because we could not demonstrate the existence of the plasmid in PaTh2. (Data not shown) The study by Girlich *et. al.* with clinical isolates from Siriraj Hospital collected around 1999 (about 4 years after the PaTh1-5 were collected) still showed that bla_{CEF-1} was prevalent. This ESBL has been present for a long while[29,30]. Results from another study also demonstrated the sequence of the 3' end of the integron element, which seemed to be identical to the integron element found in PaTh2. Therefore, there are two complete integron elements in PaTh2[31]. For the clinical point of view, the treatment options left for patients in whom these resistance isolates are obtained are very limited. Carbapenems (imipenem or meropenem) may be arbitrarily used for certain patients, because there has been one imipenem resistant strain that has emerged from one patient. (The patient from whom PaTh2 strain was originally isolated, personal observation.) In Thailand, the resistance genes collected on the integron element are not only found in *P. aeruginosa*, but also in other Gram negative rods, such as *E. coli*, *Klebsiella pneumoniae*, and other *Enterobacteria-ceae*[29,30,32]. The possibility of spread of class B β-lactamase as an integron cassette is also rising, as there have been reports from Japan, Singapore, Taiwan and Canada[33-37]. The spread of the integron elements along with conjugative plasmids and transposons is also problematic and difficult to control. There must be continuous study to monitor and a surveillance system to cope with this resistance problem, so the physicians can know the treatment options and the best alternative according to good evidence obtained from such study.

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