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Identification of R-Plasmids of *Pseudomonas aeruginosa* Isolates From Clinical Sources by Recombinant DNA Techniques

Fatih İZGÜ*, Jale US*

Summary: The R-plasmids, which transfer resistance to P. aeruginosa strains against generally used antibiotics in the treatmet of the infections caused by this gram (-) bacteria were investigated.

The P. aeruginosa samples used in this study were isolated from the clinical cases of Gülhane Military Medical Academy (GATA), such as throat, ear and urinary tract infections. Those clinical specimens of P. aeruginosa were tested for their sensitivities to fourteen different antibiotics that are used against P. aeurigonosa infections in hospitals.

It was found that ampicillin was the only antibiotic that all of the P. aeruginosa samples were resistant to. Using ampicillin as a selectable agent, plasmid isolations were done from all of the P. aeruginosa isolates.

The isolated plasmids were then digested by the restriction endonuclease Bam HI. The agarose gel electrophoretic analysis showed that all of the plasmids had only two and the same Bam HI sites.

By the fragment size analysis of the Bam HI cut plasmids, it was determined that all of the P. aeruginosa isolates, that were resistant to ampicillin, contained identical plasmids of 6.25 kb.

This result indicated that the P. aeruginosa samples used in this study carry an R-plasmid of 6.25 kb, which is not similar to those generally found R-plasmids in this species.

Key words : P. aeruginosa, R-plasmid, Antibiotic resistance.

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Klinik Vakalardan İzole Edilen Pseudomonas Aeruginosa Örneklerindeki R-Plazmidlerin Rekombinant DNA Teknikleri ile Tanısı

Özet: P. aeruginosa enfeksiyonlarında genellikle kullanılan antibiyotiklere karşı bu gram (-) bakteriye dirençlilik kazandıran R-plazmidleri incelenmiştir.

Bu çalışmada kullanılan P. aeruginosa örnekleri Gülhane Askeri Tıp Akademisindeki (GATA) boğaz, kulak ve idrar yolları enfeksiyonları klinik vakalarından izole edilmiştir. P. aeruginosa'nın bu klinik örnekleri bu bakterinin sebep olduğu enfeksiyonlarda genellikle hastanelerde kullanılan 14 değişik antibiyotiğe karşı duyarlılık testine tabi tutulmuştur. Bu antibiyotikler arasında, P. aeruginosa örneklerinin tümünün dirençlilik gösterdiği antibiyotiğin yalnızca ampisilin olduğu tesbit edilmiştir. Böylece ampisilin seçici ajan olarak kullanılmış ve P. aeruginosa örneklerinin tümünden plazmid izolasyonu yapılmıştır.

Izole edilen plazmid DNA'ları daha sonra Bam HI enzimi ile kesilmiştir. Agaroz jel elektroforezinden alınan sonuçlar plazmidlerin hepsinin bu enzim için yalnızca iki tane ve aynı kesim noktalarına sahip olduğunu göstermiştir.

Bam HI enzimi ile kesilen plazmidlerin kesit büyüklüğü analizleri yapılmış ve ampisiline dirençli olan P. aeruginosa örneklerinin hepsinin 6.25 kb büyüklüğündeki aynı plazmidi içerdikleri tespit edilmiştir.

Bu sonuca göre çalışmamızda kullanılan P. aeruginosa örneklerinin genellikle bu türde bulunan R-plasmidlerine benzemeyen 6.25 kb'lık değişik bir plazmid içerdikleri kanısına varılmıştır.

Anahtar kelimeler : P. aeruginosa, R-plazmid, Antibiyotik dirençliliği.

Introduction

Human infections caused by *Pseudomonas aeruginosa* are usually severe and difficult to treat because of the organism's resistance to many of the commonly used antibiotics[1,2].

P. *aeruginosa* infections can occur in any tissue, and body site such as lung, urinary tract, corneal tissue and wounds or burns. The organism, and so the infec-

(*) METU, Faculty of Education, Dept. of Biology Education-ANKARA.

tion, can disseminate to new sites by hematogenous route, and cause septicemia and focal lesions[3, 4].

Most of the commanly used antibiotics are not effective in the treatment of P. *aeruginosa* infections. The resistance of P. *aeruginosa* to most antimicrobial agents has allowed this organism to flourish while more susceptible organisms have been suppressed[2, 3].

Particularly, the ß-lactams are not effective against Pseudomonas. On inital isolation, the aminoglycosides (amikacin, gentamicin, and tobramycin) and the extended-spectrum penicillins (azocillin, carbenicillin, mezlocillin, piperacillin and ticarcillin) are effective on the bacteria. But, resistance develops if the duration of therapy is long, and when the penicillins are used alone. So, for the treatment of life threatening infections, combinations of high dose aminoglycosides and extended-spectrum penicillins are recommended[1, 4, 5, 6].

The most clinically significant antibiotic resistance is determined by their gene located on extrachromosomal DNA elements called plasmids[7,8]. Plasmids are extrachromosomal genetic elements that can replicate independently of the host chromosome. Although they are considered nonessential for the cell, they often perform secondary functions that are vital to the host cell under certain conditions such as surviving in medium supplemented with antibiotics. Carbenicillin, gentamicin, tobramycin, colistin are examples of antibiotics, which are active against P. aeruginosa. But widespread use of such drugs have resulted in the development of a variety of R factors (resistant factors) which can inactivate these antibiotics by phosphorylation, adenylation, acetylation or by a combination of them[7, 9, 10].

As increasing number of Pseudomonas strains harbouring different Resistant-plasmids (R-plasmids) active against such therapeutically important drugs are being isolated in different hospital conditions, the objective of this research was to investigate the characteristics of the resistance-factors in specimens obtained from Gülhane Military Medical Academy (GATA).

Materials

Bacterial Strains:

The Pseudomonas strains that were used in our study were all isolated from the clinical cases of Gülhane Military Medical Academy (GATA) such as throat, ear, and urinary tract infections.

For plasmid tranformation E. coli HBIOI (F^-, r_B^-, m_B-recA, ara, ProA, lacY, galK, str, xy15, mtl, SupE) was used.

Culture Media:

Blood agar (Mueller Hinton agar supplemented with blood) and L-borth (0.5% Yeast extract, 0.5% NaCl, 1% tryptone) were used for routine growth of the bacteria.

Brain Heart Infusion broth (Biolife), Clarc-lubs media, Urea slant medium, Simmons Citrate agar slant (oxoid), Kligler Iron agar (Scott), Mueller Hinton agar (Gibco BRL) were used for identification tests of Pseudomonas sp.

Antibiotics:

For routine uses Ampicillin Sodium Salt, (sigma), and for antibiotic sensitivity tests; Trimethoprim-sulfamethoxazole (1.25-23.75mg), gentamycin (10mg), tobramycin (10mg), tetracycline (30mg), Chloramphenicol (30mg), ofloxacin (10mg), cephaperazon (75mg), amikacin (30 mg), piperacillin (100mg), ampicillin (10mg), cephotaxime (30mg), oxalinic acid (2mg), ceftazidime (30mg), ceftriaxsone (30mg) (Oxoid, UK) discs were used.

Methods

Tests For Identifiation of Pseudomonas sp.[11, 12, 13].

Indole Test: All strains were grown overnight in 5ml of Brain Heart Infusion broth culture and 5 drops of Kovac's reagent added to each tube.

Methyl-Red Test: The bacteria were grown in 5 ml of Clark-Lubs media at 37°C for 48 hours. Then five drops of methyl-red indicator was added.

Voges Proskauer Test: The bacteria were grown in 1 ml of Clark Lubs medium at 37°C for 48 hours; then 0.2 ml of KOH (40%) and 0.6 ml of α-naphtol were added.

Urea Utilization Test: 5 ml of a urea slant medium (with phenol red as an indicator) was inoculated by the bacteria and incubated at 37°C overnight.

The Citrate Test: Bacteria were grown on 5 ml of Simmons citrate agar slant at 37°C.

Tests for Lactose Fermentation an H_2S Production: Kligler Iron slant was inoculated by the bacteria and incubated at 37°C overnight.

Test For Oxidase Enzyme: Bacteria were grown on Mueller Hinton agar and a drop of Commercial oxidase reagent (scott) was added on this medium.

Test For Catalase Enzyme: Bacteria were grown on Mueller Hinton Agar and a drop of H_2O_2 was added on to the medium.

Test For Pyocyanin Production: Bacterial strains were grown in 5 ml of Brain Hearth Infusion broth and it was left at room temperature for 1-2 weeks, then 1 ml of chloroform was added to the medium.

The Antibiotic Sensitivity Test

Pseudomonas aeruginosa sp. were grown in Brain Hearth Infusion broth culture. The surface of the Mueller Hinton agar medium was inoculated with a sterile cotton swab. After 15-20 min, each antibiotic disc were placed on a medium at particular distance from each other. After overnight incubation, zone diameters were measured.

Plasmid Isolation (Lysis By Boiling)

The method used for plasmid isolation was adapted from Holmes & Quigley (1981)[14].

Cells were grown in 5 ml of L-broth containing 5 ml ampicillin to a cell density of about A_{650} = 1.2 at 37°C. Bacteria wre then harvested at 4000 rpm in a swingout centrifuge (Nüve rotor type 815) at 4°C. Cell pellet was resuspended in STET buffer (8% sucrose, 5% triton X-100, 50mM EDTA- 50mM tris, pH:8.0) and transferred to Eppendorf microcentrifuge tubes. A 25 ml of freshly prepared lysozyme (Sigma) solution was added. After incubation at room temperature for 20 min, the tubes were placed in a boiling water both for 40 sec exactly and centrifuged immediately at 12.000 rpm (Biofuge 13, Heraeus) for 10 min at 4°C. The supernatant was extracted once with phenol (Merck) buffered with TE (10 mM tris. Cl, 1mM EDTA; pH: 7.6) and once with phenol: chloroform: isoamylalcohol (25:24:1).

The DNA was precipitated at -20°C for 20 min by adding 2 volumes of ice-cold 95% Ethanol (Merck). The plasmid DNA was collected by centrifugation at 12.000 rpm for 10 min at 40C and dessiccated to remove Ethanol and then resuspended in 10 mM TE buffer.

Gel Electrophoresis of the Plasmid DNA[15]

After each plasmid isolation, gel electrophoresis of the DNA was performed in 1 % agarose using λ virus DNA Eco RI/Hind III double digest as a standard marker in 11-14 horizantal gel tank at 50 V (power supply model 200, BRL) for 5 hours and stained in a 0.5 mg/ml Et-Br solution. The gel then placed directly on the top of a 300 nm transilluminator (Fotodyne, Foto/PrepI) and photographed (Fotodyne Paloroid camera)

Restriction Digest of the plasmid DNA:

The plasmid DNA was digested by restriction enzyme BamHI (Sigma) in a 25 µl digestion mix (2 µl DNA- 2 µl restriction buffer, 1 µl enzyme, 20 µl ddH_2O) for 1 hr at 37°C in dribath (Thermolyne Type 17600). The restriction fragments was analyzed by agarose gel electrophoresis as explained before.

Estimation of plasmid DNA Fragment Size[15]

The measurement of fragment size was done by using the mobilities of the λ Eco RI/Hind III marker digest fragments to construct a calibration curve; the sizes of the unknown fragments were determined from the distance they have migrated (Figure 1).

Transformation of P. aeruginosa Plasmid DNA to E. coli (HB101)[15]

The method used for plasmid transformation was addapted from Cohen, Chang and Hsu (1972).

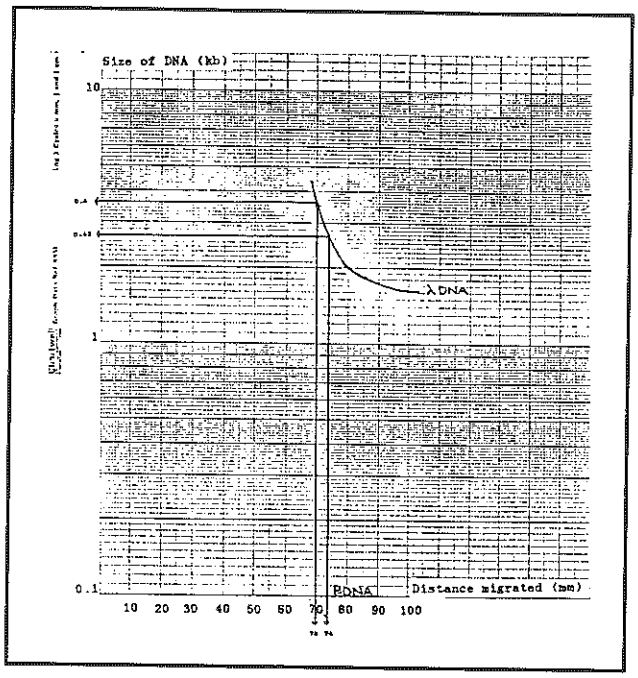

Figure 1. Standart graphical method for size estimations of the DNA fragments cut by Bam HI

A 5 ml culture of E. coli HBI01 (F^-, r_B^-, m_B^- recA, ara, ProA, lacY, galK, str, xy15, mtl, SupE) was grown overnight in L-Broth at 37°C. 1 ml of this culture was then added to 50 ml fresh L-Broth and grown for 2 hours at 37°C with vigorous agitation. Then cells were pelleted at 3000 rpm for 10 min at 4°C and resuspended in ice-cold CaCl2 (50 mM) for 10 min. The cells were again harvested (3000 rpm, 10 min, 4°C) and resuspended in 3.3 ml ice-cold $CaCl_2$.

Aliquots of 200 µl of competent cells were mixed with 100 µl of 100 mM tris, pH 7.5, and 1 µg of plasmid DNA. The mixture was left on ice for 10 min and exposed to heat shock at 42°C for 2 min. After the heat shock, the transformation mix was then added 3 ml L-Broth and incubated at 37°C for expression of the selectible marker. Cells were harvested (3000 rpm, 10 min, 20°C), and washed in STE buffer (10mM NaCl, 10 mM tris pH, 1mM Na_2EDTA, pH 8.5) and plated onto L-Broth+agar containing the appropriate selectible agent (ampicillin, Sigma) at 37°C for 16-24 hours.

Results and Discussion

Pseudomonas samples used for the present study were isolated from clinical cases such as throat, ear and urinary tract infections (Table 1)

Table 1. Clinical sources which Pseudomonas samples were obtained

Clinical Sources	Number
Throat	19
Urine	8
Ear	6
TOTAL	33

All of these strains were subjected to a series of biochemical tests and the results of these tests showed that all of the 33 isolates were Pseudomonas aeruginosa species (Table 2).

All of the isolates which were identified as P. *aeruginosa* according to the biochemical test results were then tested for their sensitivities against generally used antibiotics in the treatments of Pseudomonas infections and all P. *aeruginosa* samples have been classified for their sensitivities to antibiotics (Table 3).

As all of the P. *aeruginosa* isolates were resistant to ampicillin, plasmid isolations were done for all P. *aeruginosa* samples by using this antibiotic as a selectable agent.

In order to estimate the sizes of the isolated plasmids, these extrachromosomal DNAs were digested with the restriction endonuclease Bam HI.

The Bam HI restricted plasmid appeared as two sharp bands on the agarose gel. The size of the fragments were found to be 2.65 kb and 3.6 kb respectively (Figure 1-2). As all the plasmids have two restriction sites for Bam HI and the same fragment sizes they were found to be similar plasmids. We have seen that P. *aeruginosa* samples that were isolated from the clinical sources all carry extrachromosomal DNA of the same size (6.25 kb).

In order to check whether this plasmid was R-Plasmid or not, we have transformed this plasmid to E. coli HB101. After the transformation, the wild type HB101 which was sensitive to ampicillin was able to grow in L-Broth containing ampicillin to a concentration of 30 ul/ml. This showed that the transformed HB101 carrying the plasmid of 6.25 kb which was isolated from P. *aeruginosa* be-

Table 2. Test results for identification of Pseudomonas species

Tests	Test result	Pseodomonas spp	Comments
Gram	(-)	(+)	All bacteria appeared to be red in color after gram staining.
Indole	(-)	(+)	Production of indole from tryptonane by the help of tryptophanese enzyme was not detected because the development of a red layer on the top of culture was absent
Methyl-Red	(-)	(+)	A yellow color was observed indicating the absence of acid
Voges-proskauer	(-)	(+)	If acetylmethylcarbinol was produced, a bright red color would be developed at the surface of the medium.
Urea	(+)	(+)	Presence of urease enzyme that converts urea into ammonia was detected by the formation of red color in urea slant medium.
Lactose Fermentation	(-)	(+)	There was no color change on Kligler iron agar slant. This indicated that these bacteria can not ferment lactose
H2S Production	(-)	(+)	Any blackening of the medium at the bottom of tube were not observed
Citrate	(+)	(+)	Formation of blue color on Simmons'citrate media showed that bacteria use citrate as a carbon source.
Oxidase	(+)	(+)	Formatin of purple color after addition of commerical oxidase reagent indicated the presence of oxidase enzyme.
Catalase	(+)	(+)	After addition of H_2O_2 on the medium, rapid gas ebullition indicated the presence of catalase enzyme.
Pyocyanin	(+)	(+)	Observation of blue color is due to presence of pyocyanin. (only for P. *aeruginosa* strains)

Table 3. Classification of P. *aeruginosa* samples for their sensitivity to commonly used antibiotics

Antibiotics	Number of Resistant Bacteria	%	Number of Intermediate Bacteria	%	Number of Sensentive Bacteria	%
Trimethoprim-Sulfamethoxazole	25	75	6	18	2	6
Gentamycin	12	36	-	-	21	64
Tobramycin	5	15	2	6	26	79
Tetracycline	20	60	6	18	7	21
Chloramphenicol	15	45	4	12	14	43
Ofloxacin	3	9	-	-	30	91
Cephaperazon	6	18	1	3	26	79
Amikacin	1	3	1	3	31	94
Piperacilline-G	3	9	3	9	27	82
Ampicilline	33	100	-	-	-	-
Cephotaxime	6	18	4	12	23	70
Oxalinic Acid	12	36	-	-	21	64
Ceftazidime	2	6	-	-	31	94
Ceftriaxone	2	6	9	27	22	67

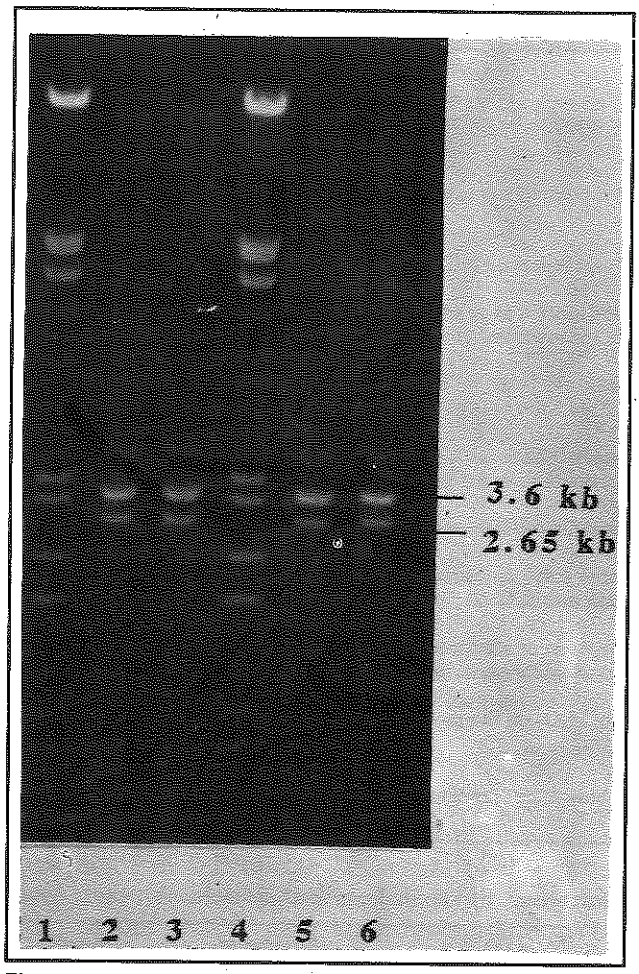

Figure 2. Agorose gel electrophoresis of Bam HI digest of the plasmid DNA. As all of the P. *aeruginosa* isolates harbour the same plasmid only four of them were shown. (Lane 1,4: λ DNA EcoRI/Hind III marker digest. Lane 2, 3, 5, 6: Plasmid DNA cut by Bam HI)

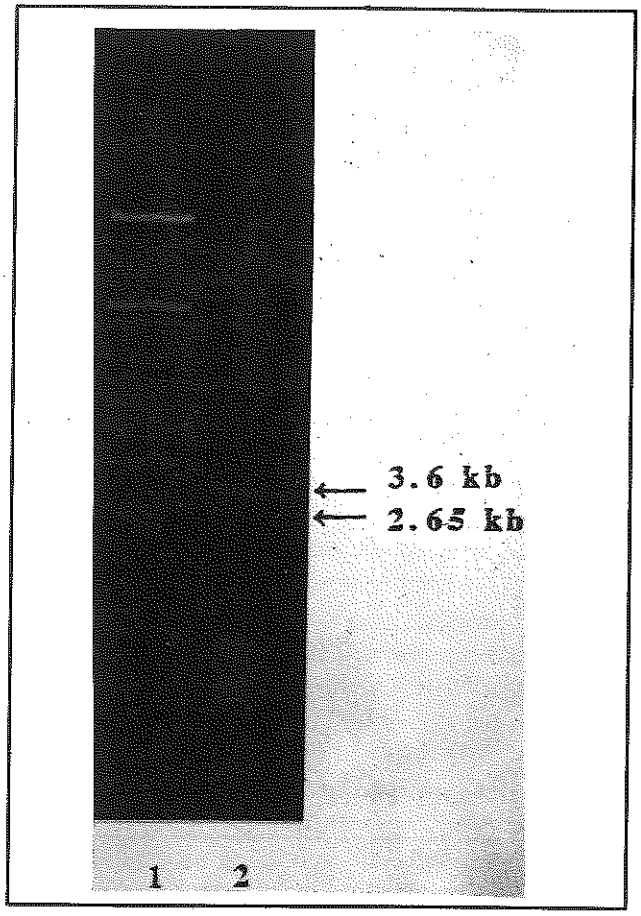

Figure 3. Agarose gel electrophoresis of Bam HI digest of the plasmid DNA isolated from E. *coli* HB101 (Lane 1: λ DNA EcoRI/Hind III marker digest. Lane 2: Plasmid DNA Bam HI digest)

came resistant to ampicillin. This result indicated that the plasmid isolated from P. *aeruginosa* was an R-plasmid.

To be sure that R-plasmid remain unchanged and replicate itself independently from the host chromosome in the E. coli HB101 strain, by using the same method for P. *aeruginosa* plasmid isolation, again we have isolated the plasmid of E. *coli* HB101 that became resistant to ampicillin after the transformation.

As a result, it was found that the plasmid isolated from ampicillin resistant E. coli HB101 remained unchanged and no recombination occured as they carried two Bam HI sites and 6.25 kb in size, as shown in gel electrophoresis (Figure 3).

At the present, because of wide spread use of chemotherapeutic preparations the R-factors spread universally for their powerfull selective advantage connected with the ability to impart resistance to many preparations.

An increasing number of P. *aeruginosa* strains having R-factors active against therapeutically important drugs are being isolated. A large amount of genetic and biological work has been done with R-plasmids of P. *aeruginosa* especially, on the P1 incompatibilitly group plasmids which are characterized by their broad host range among gram negative bacteria[16, 17 18].

The most commonly isolated R-plasmids from hospital isolates of P. aeruginosa are RP1, RP4, RP8, RP8, R68, R68-5 and RK2. Organisms harbouring these plasmids gain resistance to P, Km, tc[6, 7].

Plasmid	Drug Resistance*	Reference
RP1	ApKmTc	Iowbury et al. (1969)
RP4	ApKmTc	Data et al. (1971)
RP8	ApKmTc	Black and Girdward (1969)
R66.45	ApKmTc	Ingram et al. (1973)

Because of their more or less common origin and very similar genetic and molecular data these plasmids are thought to be closely related, if not identical[19, 20].

These plasmids carry unique restriction sites for restriction endonucleases EcoRI, Bam HI and Hind III and they are all high molecular weight circular DNA's. (>30Md)[19, 20, 21].

As these P1 in compatibility group plasmids are common in hospital isolates of P. *aeruginosa*, in this study we were also expecting, if exist, to isolate R-factors from the strains of P. *aeruginosa* that were isolated from different clinical sources from GATA hospital to be similar to those plasmids mentioned above.

In order to determine whether a given, bacterium harbours plasmids it is necessary to isolate DNA by plasmid is isolation procedures.

The result of these experiments showed that the plasmid that was have isolated was not similar to those P1 incompatibility group plasmids mentioned above as it is 6.25 kb in size and have two restriction site for the restriction endonuclease Bam HI.

Evidence is accumulating that R-plasmids can undergo variations when transferred to various bacterial strains. This appears to be due to genetic recombinations between R-factor and the components of the P. *aeruginosa* genome. The possibility of the genetic change in them during laboratory passages in bacterial strains is very high.

During physical isolation of various plasmids, both from P. *aeruginosa* and P. *putida*, small covalently closed circular molecules have been identified for which no functions are known (cryptic plasmid)[7].

In order to characterize the plasmid which was isolated from P. *aeruginosa* samples of different clinical sources, at the first step it is necessary to construct a detailed restriction map of this plasmid and secondly by plate mating tests the transferibility of this plasmid by conjugation to other enteric bacteria as E. *coli* and the stability must be determined.

The above mentioned, characterization experiments for this plasmid has been planned and started as new research project.

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* P. penicillin, Ap ampicillin, Km kanamycin, Tc tetracycline

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Medeni olmayan milletler, medeni olanların ayakları altında kalmaya mahkumdurlar.
Atatürk