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Article

Curing of Resistant *Pseudomonas aeruginosa* by Ethidium Bromide

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ABSTRACT

The *Pseudomonas aeruginosa* bacteriocin represents one of the survival methods in the mixed communities. It had its structure that gathered between colicin, which had enzymatic activity, and the pore-former toxin. Bacteriocins represent a way to gain the battle against the immune system. Antibiotics were the most effective therapy, but with time and random usage, antibiotic resistance has been developed and spread worldwide. In this study, ten isolates of *Pseudomonas aeruginosa* were detected for bacteriocin production against three *Escherichia coli* isolates. *Pseudomonas aeruginosa* isolates were bacteriocin producer. After that, curing by four concentrations (50, 75,100 and 125) µg /ml of Ethidium bromide was done. The (75 and 100) µg /ml were the most effective concentration in curing. The Antibiotic sensitivity test for the antibiotics was done before and after curing in order to make a combination between bacteriocin production and curing as a way to gain the battle against antibiotic resistance. This study proved that the anti-plasmid factor could be considered a promising way to deal with the progress of the spread of antibiotic resistance in the community.

Keywords: *Pseudomonas aeruginosa*, bacteriocin, curing and antibiotic resistance.

INTRODUCTION

Bacterial colonies were not living alone in their niches; they faced different threats from competing microorganisms. As much as the bacteria had solutions to these threats, they could survive and win the battle on the nourishment. The competing microorganisms could be related to strains genetically. One of the weapons that Pseudomonas aeruginosa (P. aeruginosa) had was bacteriocin production [1].

Bacteriocins are antibacterial proteins. Gram-negative bacteria produce to compete with the surrounding microorganisms. They affect the competing microbe's cell envelope and penetrate it; it looks like a toxin that has pore-forming activity [1].

By 2050, ten million deaths were predicted to occur; this is a picture of seven hundred deaths worldwide due to antibiotic-resistant microorganisms [2]. One of many reasons behind this growing problem is the random overuse of medicines in

human societies and the environment. 3. From the 1960s, only 2 new antibiotics were released to use, which gave a sign to global society about the depth of antibiotic resistance problem [4]. Colistin represents the last line of resistance, but fortunately, there were isolates gained from animals and humans that were resistant to this antibiotic. The reason behind this resistance may be the presence of MCR-1, encoded on a plasmid and could transmit horizontally, magnifying the fear of antibiotic resistance [5,6], in addition to the production of Extended Spectrum Beta Lactamase [7]. Identifying new resistance genes and extraordinary ways of resistance, e.g., the tetracycline efflux system of the oral metagenome [8], besides intracellular antibiotic inactivation, magnifies the global concern about antibiotic resistance [9]. The global demand was to get new antibiotics to overcome the Gram-negative bacilli or remove the resistance markers, e.g., plasmids. The aim was to detect bacteriocin production from P. aeruginosa against E. coli. Besides, curing Ps. aeruginosa with ethidium bromide, and Finally comparing their antibiotic resistance before and after curing.

MATERIALS AND METHODS

Bacterial isolates:

Three isolates of P. aeruginosa and ten clinical bacterial isolates of Escherichia coli were collected from cultures of higher studies laboratories from Mustansiriyah University –College of Science- Biology Dep. and re-identified by bacteriological and biochemical tests [10].

Detection of bacteriocin production:

The three P. aeruginosa isolates were detected for bacteriocin production against three isolates of E. coli by the Kirby-Bauer Disc diffusion Method by using Mueller-Hinton agar plates.

P. aeruginosa was cultured at 37 C for 24 hr, then centrifuged at 10 000 rpm for 10 min to get supernatant free of cells, filtered through a microfilter unit (0.22 µm) and finally stored at 4 C.

E. coli cultured agar plates; then wells were made by a sterile cork borer, then 50 µl of bacteriocin (prepared about 24 hrs) were pipetted into the wells and incubated at 37ºC for 24 hours. Each plate had five wells filled by the bacteriocin of five different P. aeruginosa isolates against one isolate of E. coli for each plate. The inhibition zones were recorded.

Curing by Ethidium Bromide:

A series of concentrations of Ethidium bromide (50, 75,100 and 125) µg /ml were prepared in Brain heart infusion broth at a final volume of 5 ml. These tubes were inoculated by 100 µl of bacteria, then grown at 37 C for 18 hr. All tubes were incubated at 37 C for 18 hr 150 rpm/ min. The growth density at tubes containing different concentrations of Ethidium bromide was compared with the control tubes. Then, the tubes that contained the highest concentration of curing agent showed an observed growth by the naked eye. Serial dilutions were made for the chosen tubes, and then 0.1 ml of each dilution was transferred to Muller Hinton Agar plates, which were used for the antibiotic sensitivity test.

Antibiotic sensitivity test:

The antibiotic sensitivity test for the *P. aeruginosa* isolates was carried out by Kirby Bauer disc diffusion [11] before and after curing by ethidium bromide. Seven different antibiotics were used to detect the antimicrobial susceptibility test, antibiotic discs consumed were from Oxoid (England), and the inhibition zones were recorded and compared with [12]. The antibiotic discs were aseptically put in

the agar using sterile forceps and then incubated at 37°C for 24 hours. The antibiotics discs used in this study were Levfloxacin (Lev,5 µg), Vancomycin (Vn, 30 µg), Amoxicillin (Am, 25 µg), Carbapenem (CRO,30 µg), Trimethoprim (T,30 µg), Erythromycin (E, 15 µg) and Nitrofurantoin (F, 30 µg).

RESULTS

After detecting bacteriocin production from P. aeruginosa against E.coli, a Curing experiment by Ethidium bromide was done.

The isolates *of P. aeruginosa* No. 2, 5 and 10 were bacteriocin producers against the three isolates of E. coli, Whereas *P. aeruginosa* No. 9 was effective against two isolates of E. coli No. 2 and 3. P. aeruginosa No. 6 is effective against two isolates of E. coli No. 1 and 2, as shown in Table 1.

***E. coli* Isolate No. spreaded on plate**	***P. aeruginosa* Isolate No. in the wells**	**Result**
E. coli 1	1, 2, 3, 4, 5	2 and 5 were producer
	6, 7, 8, 9, 10	6, 7, 8, 10 were producer
E. coli 2	1, 2, 3, 4, 5	2 and 5 were producer
	6, 7, 8, 9, 10	6, 9, 10 were producer
E. coli 3	1, 2, 3, 4, 5	2 and 5 were producer
	6, 7, 8, 9, 10	9, 10 were producer

Table 1: Effect of Bacteriocin production from *P. aeruginosa* on *E. coli*

They are not like antibiotics of a broad spectrum; the absence of selective pressure reduces the resistance formation in the microbial niches, and secondly, the killing activity is limited, which provides chances to treat bacterial infections without affecting the normal flora.

The patient's health during the usage of antibiotics depends on keeping the gut normal flora, which in turn enhances the patient's health and treatment outcome.

Four concentrations of Ethidium bromide were used (50, 75,100 and 125) µg/ml. The results showed that the (75 and 100) µg/ml were the most effective concentrations of Ethidium bromide on curing *P. aeruginosa*.

Isolate 10 changed from resistant to sensitive to Levofloxacin antibiotic. Isolate No. 8 changed from resistant to sensitive to carbapenem and Trimethoprim antibiotics and from sensitive to resistant to Nitrofurantion antibiotics.

Isolate No. 6 changed from sensitive to resistant to Levofloxacin antibiotic. The isolate No. 9 changed from sensitive to resistant to Nitrofurantion antibiotics. IsolateIsolate No. 2 was not affected ultimately, as shown in Table 2.

Beta-lactam antibiotics are one of the most used antibiotics against Gram-negative bacteria. However, antibiotic resistance appeared, leading to a fear of failure and high morbidity and mortality rates, consuming the health care system a lot of money [22].

P.aeruginosa isolate No.		Lev	Vn	Am	CRO	T	E	F
5	Before	25	R	R	R	R	R	R
	After	15	R	R	R	R	R	R
10	Before	15	R	R	R	R	R	R
	After	21	R	R	R	R	R	R
6	Before	30	R	R	R	R	R	R
	After	18	R	R	R	R	R	R
8	Before	25	R	7	10	9	6	24
	After	30	R	R	20	R	R	R
2	Before	25	R	R	23	R	R	R
	After	25	R	R	20	R	R	R
9	Before	25	R	9	8	6	R	25
	After	30	R	R	R	R	R	R

Table 2: Antibiotic sensitivity test before and after curing by ethidium bromide of *Pseudomonas aeruginosa* Levfloxacin (Lev,5 µg), Vancomycin (Vn, 30 µg),Amoxcillin (Am, 25 µg), Carbapenem (CRO,30 µg), Trimethoprim (T,30 µg), Erythromycin (E, 15 µg) and Nitrofurantion (F, 30 µg).

The most potent antibiotics were the carbapenems, which represent the last choice for physicians [23]. The genes coding for Metallo-Beta Lactamase were found on plasmids; this means horizontal gene transfer into numerous surrounding bacteria. The curing of bacteria led to the change of bacteria from resistant to susceptible. Five agents acted physically or chemically to remove plasmids (e.g., ethidium bromide, acridine orange, sodium dodecyl sulfate, pH changes and high temperature) [24].

Some of these curing genes worked unspecificly, leading to cell damage; multiple antibiotic resistance could be affected but could not be predicted by action [25].

They proved ethidium bromide caused a more significant elimination rate than Sodium Deo Sulphate and acridine orange dye [24].

Antibiotic resistance markers are found either on chromosomes, plasmids, integrons and transposons [25]. The antibiotic resistance markers found on plasmid represent a significant player in resistance markers transfer between different bacteria [27].

The plasmid curing proved the intimate connection between antibiotic resistance and plasmid presence [28]. After curing resistant *Acinetobacter baumanii* and *P. aeruginosa*, it became sensitive; this proved that antibiotic resistance markers of these bacteria were plasmid-mediated.

DISCUSSION

The Pseudomonads represent a genus of bacteria with tremendous metabolic capabilities to live and compete with microorganisms, which may be opportunistic

pathogens. These bacteria enhance plant growth or pollutant degraders in the environment [13]. In order to do that, they were supplied with strong substances with different structure and activity. One was Bacteriocins, which had antibacterial activity and acted specifically on the related bacteria [14]. The bacteriocins of *P.aeruginosa* were named pyocins, and their benefit for the producer strain was Ecoevolutionary [15]. These points were like *E. coli* colicins [16]. The aim of bacteriocin docking onto cells was outer membrane proteins (OMPs), which were responsible for iron uptake (by siderophores) [17] because of the structure resembling *P. aeruginosa* Pseu Ms and *P.syringae* and *E. coli* colicin; they proposed that they shared the same ancestry and act on the same site of the components of cell surface [18]. Besides the potent activity of bacteriocins, they had an important feature: the specificity of affected bacterial species of these molecules, which served two aims [19]. Antibiotic-associated diarrhea and *Clostridium difficile* infection were found due to dysbiosis after antibiotic use [20]. Microbial imbalances have been proposed to cause chronic diseases (e.g., diabetes, Crohn's disease, rheumatoid arthritis and obesity) [21] and the last decade witnessed attempts from pharmaceutical companies that included a post-genomic program of antibiotic discovery, which resulted in minimum success and few antibiotics released to the market. The antibiotics recently used in the clinical field appeared after naturally occurring antibiotics were detected and modified. This led to a return to natural products, which became safer than new antimicrobial agents [19].

CONCLUSIONS

The study proved that ethidium bromide was one of the suitable solutions for plasmids curing, but its use in therapy was not acceptable due to mutagenicity and toxicity. This made the scientists change their concern to a new, non-toxic, and active curing agent that could be used in addition to antibiotics to get a solution for antibiotic resistance, besides factors that induce a shift in the plasmid copy number.

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