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Research Paper / Araştırma Makalesi

Pseudomonas fluorescens Isolation from Green Salads and Antibiotic Susceptibilities of Isolates

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ABSTRACT

Currently, leafy green salads are often consumed because they are considered as practical and healthy. However, during their preparation, both inadequate washing and contact with non-hygienic surfaces may increase their microbial load. This may cause several health problems for individuals consuming salads. The overuse of antibiotics has led to the emergence of multidrug resistant bacteria, including foodborne pathogens. Biofilm production ability of these pathogenic bacteria makes it difficult to treat infections caused by these pathogens. The aim of this study is to isolate and identify *P. fluorescens* from leafy green salads collected from different restaurants. A total of 72 isolates were isolated from leafy green salads, and 29 of these isolates were identified by PCR as Pseudomonas and 9 of them identified as *P. fluorescens*. All *P. fluorescens* isolates were resistant to ampicillin, amoxicillin, cefuroxime, ceftazidime and ceftriaxone antibiotics. The results of this study showed that additional attention for the hygiene conditions is needed during the preparation and storage stages of leafy green salads.

Keywords: Leafy green salad, *Pseudomonas fluorescens,* Antibiotic resistance

Yeşil Salatalardan *Pseudomonas fluorescens* İzolasyonu ve İzolatların Antibiyotik Duyarlılıkları

ÖZ

Günümüzde yeşil salatalar genellikle pratik ve sağlıklı olduklarını düşünerek tüketilmektedir. Ancak salataların hazırlanması sırasında, salata malzemelerinin yetersiz yıkanması ve hijyenik olmayan yüzeylerle teması, salataların mikrobiyal yükünü arttırmaktadır. Bu durum salataları tüketen bireyler için sağlık sorunlarına neden olabilir. Antibiyotiklerin aşırı kullanımı, gıda kaynaklı patojenler dahil olmak üzere çoklu ilaç direncine sahip bakterilerin ortaya çıkmasına neden olmuştur. Bu patojen bakterilerin biyofilm üretme özelliklerinin olması, bu patojenlerin sebep oldukları enfeksiyonların tedavisini zorlaştırmaktadır. Bu çalışmanın amacı, farklı restoranlardan temin edilen yeşil salatalardan *P. fluorescens* izolasyonu ve tanımlanmasıdır. Yeşil salatalardan toplam 72 izolat izole edilmiş ve bu izolatların 29'u PZR ile Pseudomonas olarak ve 9'u *P. fluorescens* olarak tanımlanmıştır. Tüm *P. fluorescens* izolatlarının ampisilin, amoksisilin, sefuroksim, seftazidime ve seftriakson antibiyotiklerine dirençli olduğu bulunmuştur. Bu çalışmanın sonuçları, yeşil salataların hazırlanması ve depolanması sırasında hijyen koşullarına daha fazla dikkat edilmesi gerektiğini göstermektedir.

Anahtar Kelimeler: Yeşil salata, *Pseudomonas fluorescens,* Antibiyotik direnci

INTRODUCTION

Over the last few years, there is an increased demand for consumption of salads [1, 2]. As leafy green salads do not need cooking and further preparation before consumption, they could potentially contain pathogens that form part of their microflora [3]. Fresh vegetables can become contaminated by pathogens at any point, from farm to fork. Nowadays due to the changes in the human lifestyle leafy green salads are mainly preferred, however they contain pathogenic microorganisms that can cause foodborne diseases [1, 4, 5].

P. fluorescens are widespread in the environment more than *P. aeruginosa* and found in refrigerated food products where its psychrotrophic character gives it the possibility to grow in the relative absence of competitors [6, 7]. It has been considered that food-derived *P. aeruginosa* and *P. fluorescens* species can cause various health problems in terms of public health [8]. However, while far less virulent than *P. aeruginosa*, *P. fluorescens* can cause opportunistic infections in humans. The most common site of *P. fluorescens* infection is the bloodstream [9, 10].

Excessive use of antibiotics has led to the emerging evolution of antibiotic-resistant bacteria [11-13]. And bacterial biofilms are inherently resistant to antibiotics.

Pseudomonas spp. members are naturally resistant to beta-lactam group antibiotics and can easily develop resistance to antibiotics thanks to their various properties. Furthermore, the treatment of infections caused by bacteria with multiple antibiotic resistance takes longer, patients need to stay in hospital longer and the mortality rates increase due to these infections [14]. Although *P. aeruginosa* is the most studied of the genus Pseudomonas, there are a limited number of studies about the opportunistic pathogen, *P. fluorescens*. It is also important to investigate the antibiotic resistance of *P. fluorescens* isolates, as they are one of the species frequently isolated from environmental samples and are closely related to public health.

In this study, *P. fluorescens* isolated and identified from leafy green salad samples by using 16S rRNA PCR method. The susceptibility of *P. fluorescens* isolates to amikacin, tetracycline, ceftazidime, cefuroxime, cefepime, ciprofloxacin, ampicillin, amoxicillin, chloramphenicol, sulfamethoxazole, ceftriaxone, and kanamycin and biofilm formation capacities were investigated.

MATERIALS AND METHODS

Culture Media and Strains

Cetrimide agar (Oxoid), plate count agar (PCA, Lab M), Müller-Hinton Agar (Merck) and Luria-Bertani (LB, Lab M) agar were used in the study. The bacterial strains used in the studies were obtained from the bacterial collection of the Department of Biology of Suleyman Demirel University.

Isolation of *Pseudomonas fluorescens* from Salads

For the study, 72 raw salad samples collected from 24 different restaurants in various districts and centers of Isparta, Turkey. Samples were stored at +4°C until analysis. 10% (w/v) peptone water was used for the sample dilution. Each different dilution was propagated to the PCA medium and to the cetrimide agar medium. The PCA medium was incubated at 35°C and for 24-48 hours. The cetrimide agar medium was incubated at 35°C for 48 hours. As a result of incubation, colony count was recorded. The isolates were stored at -20°C until use.

Phenotypic Determination of Isolates

Gram staining, catalase test, oxidase test, and motility test were applied for all isolates and the results were evaluated.

Polymerase Chain Reaction of Isolates (PCR)

Lysis buffer containing 0.25% SDS and 0.05 N NaOH was used for DNA isolation [15]. 1-2 colony bacteria were added to 50 µL of lysis buffer and boiled for 15 minutes. After cooling the tubes at room temperature, they were centrifuged at 13,000 rpm for 2 minutes. The supernatant was used for PCR. Primers were purchased from Iontek (İstanbul, Turkey).

The nucleotide sequences of primers for 16S rRNA for Pseudomonas genus are below with a product size 614 bp [15].

PA-GS-F GACGGGTGAGTAATGCCTA
PA-GS-R CACTGGTGTTCCTTCCTATA

PCR products were analysed using 1.4% agarose gel and Sybr green. 100-1000 bp DNA marker was used. After electrophoresis, the agarose gel was examined in the UV imaging system and PCR products of 614 bp were recorded as belonging to the genus Pseudomonas.

PCR was performed for the *rpoS* gene (product size 142 bp) to determine the *P. fluorescens* of isolates identified as Pseudomonas. Nucleotide sequence of primers used is:

F- CAAAGGACTATAACAATGGCTCTCAG
R- ATTTGGTGCGAACGGAAGGTGGAGTT

Investigation of Antibiotic Susceptibilities of Isolates

The antibiotic susceptibilities of isolates identified as *P. fluorescens* were investigated by Kirby-Bauer disk diffusion method according to CLSI (Clinical and Laboratory Standards Institute) [16]. *P. fluorescens* suspension was adjusted to match the tube of 0.5 McFarland turbidity standard which equals to 1.5×10^8 colony-forming units (CFU)/mL and,

suspension was applied to the Mueller-Hinton (MH) agar. Then, antibiotic discs were placed and incubated at 30°C for 24 hours. The antibiotics used in the study were obtained from Bioanalyse (Table 1.).

At the end of the incubation, the inhibition zone diameters around the antibiotic discs were measured and recorded. The results were determined as sensitive (S), moderate (I) and resistant (R) according to standards of European Committee on Antimicrobial Susceptibility Testing [17, 18].

Table 1. Antibiotics used in the study for the antibiogram

Antibiotic Group	Antibiotic Name	Antibiotic Abbreviation	µg/antibiotic disc content
Penicilline	Amoxicilline	AMC30	30
	Ampiciline	AMP10	10
Cephalosporin	Cepefime	FEP30	30
	Ceftazidime	CAZ30	30
	Ceftriaxone	CRO30	30
	Cefuroxime	CXM30	30
Fluoroquinolone	Ciprofloxacin	CIP5	5
Aminoglycoside	Amikacin	AK30	30

Biofilm Test

For the biofilm test, the method described by [19] was used. The identified *P. fluorescens* samples were grown in LB medium at 30°C for 18-20 hours. 1 mL of the bacterial culture (0.5 McFarland density), was incubated at 30°C for 48 hours. At the end of the incubation, the cultures were poured and the tubes were washed 3 times with sterile purified water. After drying the tubes at room temperature, they were stained with 1 mL 0.1% (w/v) crystal violet for 30 minutes and the excess of the dye was washed with sterile purified water. 1 mL 95% (v/v) ethanol added to the tubes and incubated 15 minutes. And the optical density of ethanol was measured at 570 nm and the amount of biofilm was analysed.

Statistical Analysis

Data represent the mean (± standard deviation, SD) of three independent experiments, each performed in triplicates.

RESULTS AND DISCUSSION

Microbiological Properties of Raw Salad Samples

Total aerobic and mesophilic bacteria were determined from 72 salads that was collected from 24 different restaurants. The total bacterial count was obtained in the values ranging from log CFU/g 4.2-8.2, while in the cetrimide agar it was found between log CFU/g 2.5-6.5.

Genotypic Analysis of Isolates

In terms of phenotypic properties, 16S rRNA PCR was applied for 72 isolates with Gram (-), catalase (+), oxidase (+), and motility test (+). After this PCR process, gel electrophoresis was performed and 25 isolates with a product size of 614 bp were recorded as Pseudomonas species.

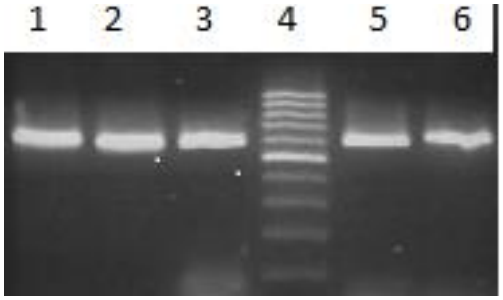

Figure 1. Agarose gel electroprohesis image of 614 bp PCR products showing that 1, 2, 3 and 6 isolates belong to Pseudomonas genus. 4, M (marker 1kb), *P. aeruginosa* ATCC 27853 (positive control) indicated by 5.

16S rRNA PCR was made from the isolates of the Pseudomonas genus using the *P. fluorescens* species spesific primers. And, 9 isolates, identified as 142 bp in product size, were recorded as *P. fluorescens.*

Investigation of Antibiotic Susceptibility of Isolates

As a result of PCR, nine isolates determined as *P. fluorescens* were examined by using disc diffusion method. The 9 *P. fluorescens* isolates were tested for susceptibility to eight antimicrobial agents by disc diffusion method. The comparison results on antibiotic susceptibilities are shown in Table 2.

All nine isolates identified as *P. fluorescens* were found to be amikacin-sensitive only while ampicillin, amoxicillin, cefuroxime, ceftazidime, and ceftriaxone were resistant (Table 2.). Cefuroxime (2nd generation), ceftriaxone (3rd generation), ceftazidime (3rd generation), all nine isolates and cefepime (4th generation), which are used as anti-pseudomonal drugs, is a remarkable result.

Table 2. Resistance of *P. fluorescens* isolates to different antibiotics

Isolate No	AMC30	AM10	CXM 30	CAZ30	CRO	FEP30	CIP5	AK 30
2G	R	R	R	R	R	R	S	S
3H	R	R	R	R	R	R	S	S
3K	R	R	R	R	R	R	S	S
16A	R	R	R	R	R	R	S	S
17A	R	R	R	R	R	R	R	S
20A	R	R	R	R	R	R	R	S
20C	R	R	R	R	R	S	S	S
23A	R	R	R	R	R	S	S	S
24B	R	R	R	R	R	S	R	S

In vitro biofilm formation properties of *P. fluorescens* isolates were evaluated. Isolates with 3H and 3K were found to have the highest biofilm formation capacity. In addition, it was determined that the biofilm production capacity of the 2G, 16A and 23A isolates were lower than the other isolates (Figure 2.).

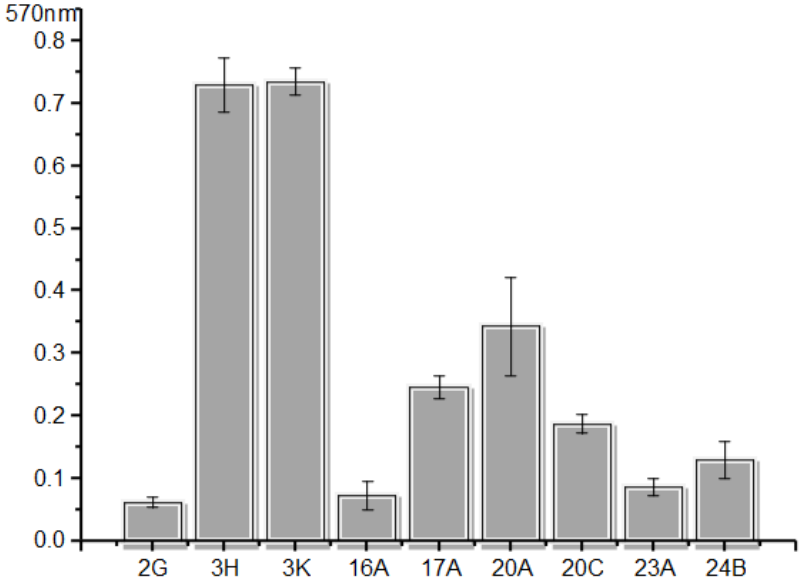

Figure 2. Biofilm forming capacities of *P. fluorescens* samples isolated from salad samples

The emergence of microorganisms with multiple drug resistance is a serious global health problem [20]. The extensive use of antibiotics in agriculture and medicine increases the number of resistance genes and the bacteria harboring them by encouraging the environmental propagation of resistance [21, 22]. These genes and microorganisms are discharged into environmental segments through human and animal waste such as manure, sewage sludge and waste water [23]. In this way, simultaneous release of antibiotics and other selective agents promotes the selection of organisms containing resistance genes [24-26].

Resistant bacteria, especially pathogens, may cause contamination during the processing of food products. Due to the minimum treatment of raw salad vegetables, it preserves its natural microflora to a great extent [27]. Raw vegetable products are often contaminated during storage, and the longer storage time increases the microbial load. It is known that Pseudomonas genus dominates in the field of lettuce and other plants that grow in the field based on culture-dependent and independent analyzes [28-30]. *P. fluorescens* is one of the most isolated microorganism from raw vegetable salads, and food-borne infections, as well as food-detrimental properties of these organisms, pose a risk to public health. It is known to be associated with some infections such as septicemia, catheter related bacteremia [31].

In this study, the susceptibility of *P. fluorescens* isolates to 8 antibiotics of different classes was investigated. All isolates were identified as resistant to at least two antibiotics of multiple chemical classes [32, 33](Table 2). All nine isolates identified as *P. fluorescens* were found to be only amikacin-sensitive while ampicillin, amoxicillin, ceftriaxone, chloramphenicol, ceftazidime and cefuroxime were resistant. All of nine isolates determining to cephalosporin, cefuroxime, ceftriaxone, ceftazidime that it is resistant is an important result.

Another important factor contributing to antibiotic resistance is the ability of microorganisms to form biofilms on both biotic and abiotic surfaces [34]. In this study, it was determined that *P. fluorescens* isolates had the capacity to produce biofilm at different rates.

CONCLUSION

The results showed that, bacteria was isolated and had multiple antibiotic resistant characteristics. Considering that plants do not come into direct contact with antibiotics, the resistance observed among the examined bacteria is alarming. Furthermore, the potential spread of antibiotic resistance through the food chain carries the risk of public health because of the potential for antibiotic resistant foodborne pathogens to be transferred to humans. While the spread of these resistance determinants increase with human activity, its quite dramatic that this situation is threaten human health in worldwide. Therefore the potential of the spread of resistant bacteria the preparation, preservation and packaging of vegetables and fruits should be applied more carefully.

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