Investigation of proteolytic, lipolytic activities and antibiotics susceptibility of some *Pseudomonas* bacteria isolated from raw milks

Çiğ sütlerden izole edilen bazı *Pseudomonas* bakterilerinin proteolitik, lipolitik aktivitelerinin ve antibiyotik duyarlılıklarının araştırılması

Tuba ÇAYLAK-TAŞ[1], Gökçen YUVAL-ÇELİK[2], Dilşad ONBAŞILI[2]

ÖZET

Amaç: *Pseudomonas* bakterileri proteaz ve lipaz gibi yüksek sıcaklıklarda kararlı ekstraselüler enzimleri sentezlerler. Bu nedenle *Pseudomonas* türleri çiğ veya pastörize sütte bozulmadan sorumlu en yaygın organizmalardır. Bu çalışmanın amacı, çiğ sütlerden izole edilen 15 adet *Pseudomonas* suşunun antibiyotik duyarlılığı, proteolitik ve lipolitik aktivitelerini belirlemektir.

Yöntem: Bu çalışmada incelenen 15 adet *Pseudomonas* spp. suşu Türkiye'de Kayseri ve Niğde illerinden toplanan 50 adet çiğ süt örneğinden izole edilmiştir. Örnekler, laboratuvara transfer edilene kadar düşük sıcaklıkta muhafaza edilmiş ve 24 saat içerisinde analize alınmıştır. İzole edilen suşlar Analitik Profil İndeks (API 20 NE) kullanılarak tanımlanmıştır. *Pseudomonas* suşlarının proteolitik ve lipolitik aktiviteleri Skim Milk Agar (SMA) ve Tribütirin Agar (TA) besiyerlerinde test edilmiştir. Bir gecelik kültürlerden besiyerlerine nokta ekim yapılmıştır. İnkübasyondan sonra SMA ve TA besiyerlerinde oluşan zonlar kumpas ile ölçülmüştür. Aynı zamanda, suşların yedi antibiyotiğe karşı duyarlılıkları test edilmiştir. *Pseudomonas* spp suşlarının antibiyotiklere karşı duyarlılığı ampisilin (10 µg), amikasin (30 µg), gentamisin (10 µg), oflaksasin (5 µg), tetrasiklin (30 µg), kloramfenikol (30 µg) ve sefuroksim (30 µg) antibiyotikler için disk difüzyon yöntemi kullanılarak belirlenmiştir. Sonuçlar CLSI standartlarına göre yorumlanmıştır.

ABSTRACT

Objective: *Pseudomonas* bacteria secrete extracellular enzymes, extremely stable to high temperatures such as protease and lipase. Accordingly, *Pseudomonas* species are the most common organisms in raw or pasteurized milk at the time of spoilage. The aim of this study was to determine antibiotic sensitivity, proteolitic and lipolytic activity of fifteen *Pseudomonas* spp. strains isolated from raw milk samples.

Method: In the study, 15 *Pseudomonas* spp. strains were isolated from 50 raw milk samples collected from Kayseri and Nigde provinces in Turkey. The samples were maintained at low temperature during transfer to the laboratory and analyzed within 24 hrs. Isolated strains were identified by using Analytical Profile Indeks (API 20 NE). Proteolytic activities and lipolytic activities of *Pseudomonas* strains were tested in Skim Milk Agar (SMA) medium and in Tributyrin Agar (TA) medium. Overnight cultures were spot inoculated onto media. After incubation, the transparent zones of bacteria on the SMA and TA media were measured by calper rule. Also, the *Pseudomonas* spp. strains were tested for their susceptibility to seven antibiotics. Antibiotic susceptibility tests of *Pseudomonas* spp. strains to ampicillin (10 µg), amikacin (30 µg), gentamicin (10 µg), oflaxacin (5 µg), tetracyclin (30 µg), chloramphenicol (30 µg), cefuroxime (30 µg) were determined by using the disc diffusion method. The results were described according to CLSI standards.

[1] İstanbul Kriminal Polis Laboratuvarı Müdürlüğü, İSTANBUL
[2] Erciyes Üniversitesi, Eczacılık Fakültesi, Farmasötik Biyoteknoloji Anabilim Dalı, KAYSERİ

İletişim / Corresponding Author : Gökçen YUVALI-ÇELİK
İstanbul Kriminal Polis Laboratuvarı Müdürlüğü, İSTANBUL
Tel : +90 352 207 66 66 / 28400 E-posta / E-mail : gycelik@erciyes.edu.tr

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Bulgular: Proteolitik aktiviteye sahip olduğu saptanan 14 *Pseudomonas* suşunun bu aktivitelerinin 14,2 ve 55,0 mm zon çapları arasında olduğu bulunmuştur. Lipolitik aktivite gösteren 13 suşun aktivitelerinin 5,3 ve 29,3 mm zon çapları arasında olduğu bulunmuştur. Test edilen *Pseudomonas* suşlarının %57'sinin antibiyotiklere karşı duyarlı, %35'inin dirençli ve %8'sinin orta duyarlı olduğu belirlenmiştir.

Sonuç: Çalışmamız ilaç sanayi ve diğer sektörlerde kullanılan mikrobiyal enzimlerin saflaştırılması ve ekonomik faydalarına yönelik konulara ışık tutacaktır.

Anahtar Sözcükler: *Pseudomonas* spp., tanımlama, antibiyotik duyarlılığı, proteolitik aktivite, lipolitik aktivite

Results: Fourteen *Pseudomonas* strains were determined that had proteolytic activities. Proteolytic activities of these strains were found between 14.2 and 55.0 mm zone diameters. Thirteen strains showed lipolytic activities. Lipolytic activities of these strains were found between 5.3 and 29.3 mm zone diameters. It was determined that 57% of *Pseudomonas* strains were susceptibility, 35% were resistant and 8% were intermediate susceptibility against antibiotics.

Conclusion: Our study shed light on purification and economic benefits of microbial enzymes used in the pharmaceutical industry and other sectors.

Key Words: *Pseudomonas* spp., identification, antibiotic susceptibility, proteolytic activity, lipolytic activity

INTRODUCTION

Refrigerated storage of milk is a common practice to control spoilage caused by the growth of mesophilic bacteria. However, psychrotrophic bacteria can grow readily at refrigeration temperatures. Psychrotrophic bacteria are ubiquitous in nature, with soil, water, plants and animals as their natural habitats (1). During storage under low temperature the raw milk undergoes spoilage due to the activity of proteinases and lipases released by psychrotrophic bacteria (2, 3).

Pseudomonas species are known as the most common spoilage organisms found in raw or pasteurized milk (2). *Pseudomonas* bacteria secrete extracellular enzymes such as protease and lipase which are extremely stable at high temperatures. Proteolytic and lipolytic activities of the psychrotrophs especially activities of *Pseudomonas* species, are valuable tools for the detection of spoilage of refrigerated foods and in assessing the shelf life of the foods (4). Proteolytic enzymes induce degradation of casein which is evident from the greyish colour and bitter taste of the milk. Lipases are responsible for degradation of milk fat associated with the development of a rancid and soapy flavour and occasionally a somewhat bitter taste due to release of low-molecular fatty acids (5).

Microorganisms move easily between ecosystems: from humans and animals to soil and water and/or vice versa. It has been reported that antibiotic resistance genes acquired by organisms in one ecosystem can easily be transfered among organisms in various ecosystem by this way. In addition, there is a greater global mobility of living organisms facilitating the spread of microorganisms and their genes around the world (6). Foodborne bacteria including known pathogens and commensal bacteria display an extensive and diverse range of resistance to antimicrobial agents of human and veterinary importance (7). Any further spread of resistance among bacteria in food is likely to have an influence on public health (8).

In view of this, 15 *Pseudomonas* strains were isolated from raw milk samples and identified using of analytical profile index (API 20 NE) in the present study. The antibiotic sensitivity, proteolytic and lipolytic activities of these strains were also investigated.

MATERIALS AND METHODS

Sample collections: Raw milk samples were collected from various villages in the provinces of Kayseri and Nigde in Turkey between 01/03/2008 and 05/25/2009. The samples were maintained at low temperature during transfer to the laboratory and analyzed within 24 h.

Isolation and Identification of Bacteria: Fifty raw milk samples were homogenized by vigorous shaking and diluted in sterile physiological water. Homogenized samples were diluted serially from 10^{-1} to 10^{-7} and then 0.1 mL of the samples and/or dilutions were inoculated on McConkey agar (Merck 1.05465) plates. Afterwards inoculated plates were incubated at 37°C for 24 h. At the end of incubation period lactose (-) colonies on McConkey Agar were picked up and inoculated onto the *Pseudomonas* CFC agar media (Merck 1.07620). Inoculated media were incubated at 37 °C for 24-48 h. After incubation Gram staining and examination under a microscope were performed on randomly selected colonies (9). The isolated bacteria were evaluated firstly according to their colony morphology, the Gram-stain, catalase activity, ability to grow at +40°C and + 42°C. Further identification of *Pseudomonas* sp. at species level was performed by using analytical profile index (API 20 NE). Identified strains were preserved in 15% (v/v) glycerol at -20°C until use.

Antibiotic Susceptibility: In the antibiotic susceptibility study; Mueller-Hinton Agar (MHA) (LAB039) medium and as antibiotics: ampicillin (10 µg), amikacin (30 µg), gentamicin (10 µg), oflaxacin (5 µg), tetracycline (30 µg), chloramphenicol (30 µg), cefuroxime (30 µg) (Bioanalyse) were used. The antibiotic susceptibility tests of *Pseudomonas* spp. strains were determined by using disc diffusion method. The results were described according to CLSI standards.

Proteolytic and lipolytic activity of *Pseudomonas* strains: Proteolytic and lipoliytic activities of *Pseudomonas* strains were tested in Skim Milk Agar (SMA) (Merck 1.15338) and Tributyrin Agar (TA) (Merck 1.01957) media, respectively. Overnight grown cultures were spot inoculated onto the media. After incubation at 37 °C for 24 h, the transparent zones of bacteria on the SMA and TA media were measured by calper rule (10).

RESULTS

In this study, total of 15 strains were isolated from raw milk and identified by using API 20 NE (Table 1). These isolates were identified in the species *Pseudomonas* fluorescens ssp. *indologenes*, *Pseudomonas vesicularis*, *Pseudomonas luteola* and *Pseudomonas aeruginosa*.

Table 1. *Pseudomonas* species isolated from raw milk and their geographical origin

Number	Strain	Species	Origin
1	T_1	*P. luteola*	Kayseri Province
2	T_2	*P. vesicularis*	Niğde Province
3	T_3	*P. vesicularis*	Niğde Province
4	T_4	*P. vesicularis*	Kayseri Province
5	T_5	*P. aeruginosa*	Niğde Province
6	T_6	*P. fluorescens* ssp. *indolegenes*	Niğde Province
7	T_7	*P. fluorescens* ssp. *indolegenes*	Niğde Province
8	T_8	*P. fluorescens* ssp. *indolegenes*	Niğde Province
9	T_9	*P. fluorescens* ssp. *indolegenes*	Niğde Province
10	T_{10}	*P. fluorescens* ssp. *indolegenes*	Niğde Province
11	T_{11}	*P. fluorescens* ssp. *indolegenes*	Niğde Province
12	T_{12}	*P. fluorescens* ssp. *indolegenes*	Niğde Province
13	T_{13}	*P. fluorescens* ssp. *indolegenes*	Niğde Province
14	T_{14}	*P. fluorescens* ssp. *indolegenes*	Niğde Province
15	T_{15}	*P. fluorescens* ssp. *indolegenes*	Niğde Province

The *Pseudomonas* strains were tested for their susceptibility to ampicillin, amikacin, gentamicin, oflaxacine tetracyclin, chloramphenicol and cefuroxime. The results of the disc diffusion assays are shown in Table 3. It was found that 57% of *Pseudomonas* strains were susceptible, 35% were resistant and 8% of them showed intermediate susceptibility against seven antibiotics (Table 2). This study revealed that while investigated *Pseudomonas* strains showed high susceptibility against amikacin, oflaxacin, gentamicin antibiotics, they were resistant against ampicillin and cefuroxime.

Table 2. Antibiotic resistance of *Pseudomonas* strains

***Pseudomonas* strains**	Antibiotics						
	AM	**TE**	**AK**	**OFX**	**CN**	**C**	**CXM**
P. luteola T_1	R	I	S	S	S	S	R
P. vesicularis T_2	R	S	S	S	S	S	R
P. vesicularis T_3	R	S	S	S	R	S	R
P. vesicularis T_4	R	S	S	S	R	S	R
P. aeruginosa T_5	R	R	S	S	S	I	R
P. fluorescens ssp. *indolegenes* T_6	R	R	S	S	S	R	R
P. fluorescens ssp. *indolegenes* T_7	R	R	S	S	S	R	R
P. fluorescens ssp. *indolegenes* T_8	R	R	S	S	S	I	R
P. fluorescens ssp. *indolegenes* T_9	R	I	S	S	S	I	R
P. fluorescens ssp. *indolegenes* T_{10}	R	I	S	S	S	I	R
P. fluorescens ssp. *indolegenes* T_{11}	R	S	S	S	S	S	R
P. fluorescens ssp. *indolegenes* T_{12}	I	S	S	S	S	R	R
P. fluorescens ssp. *indolegenes* T_{13}	R	S	S	S	S	S	R
P. fluorescens ssp. *indolegenes* T_{14}	R	S	S	S	S	R	R

AM: Ampicillin TE: Tetracycline AK: Amikacin OFX: Ofloxacin
CN: Gentamicin C: Chloramphenicol CXM: Cefuroksime
R: resistant, S: susceptibility, I: intermediate-susceptibility

Proteolytic activities of *Pseudomonas* isolates were stated in Table 3. It was determined that 14 strains had proteolytic activities. Proteolytic activities of these strains were found between 14.2-55.0 mm with an average 28.1 mm zone diameter. The highest proteolytic activity of 55.0 mm was found in *P. fluorescens* ssp. *indologenes* T7 strain. The lowest proteolytic activity (14.2 mm) was determined in *P. fluorescens* ssp. *indologenes* T11 strain. (Table 3).

Table 3. Zone diameters (mm) obtained for proteolytic and lipolytic activities of *Pseudomonas* strains

***Pseudomonas* strains**	*** Zone diameters (mm)	
	Proteolytic activity	Lipolytic activity
P. luteola T_1	-	-
P. vesicularis T_2	36.3±2.9	5.3±0.2
P. vesicularis T_3	25.7±4.8	9.1±0.4
P. vesicularis T_4	34.8±8.9	11.1±0.8
P. aeruginosa T_5	21.0±0.0	7.5±0.1
P. fluorescens ssp. *indolegenes* T_6	16.0±0.0	12.2±1.5
P. fluorescens ssp. *indolegenes* T_7	55.0±14.1*	21.8±4.9
P. fluorescens ssp. *indolegenes* T_8	41.4±16. 2	29.3±3.0*
P. fluorescens ssp. *indolegenes* T_9	53.9±7.8	14.9±2.4
P. fluorescens ssp. *indolegenes* T_{10}	46.1±10.0	25.2±0.6
P. fluorescens ssp. *indolegenes* T_{11}	14.2±5.7	19.8±0.7
P. fluorescens ssp. *indolegenes* T_{12}	23.5±2.4	21.2±5.4
P. fluorescens ssp. *indolegenes* T_{13}	29.2±3.1	13.0±3.9
P. fluorescens ssp. *indolegenes* T_{14}	15.4±0.6	-
P. fluorescens ssp. *indolegenes* T_{15}	26.3±0.9	9.4±0.6
Average	28.1±5.16**	15.4±1.6**

-: Not determined.
* The highest proteolytic and lipolytic activities zone diameter
** The average proteolytic and lipolytic activities zone diameter
*** Values are the means±standard deviations of triplicate measurements.

Lipolytic activity of *Pseudomonas* spp. strains are also stated in Table 3. Lipolytic activities were tested in Tributyrin Agar (TA) medium. It was found that 13 strains had lipolytic activities. Lipolytic activities of these strains were found between 5.3-29.3 with an average 15.4 mm zone diameter. The highest zone diameter of lipolytic activity 29.3 mm was found in *P. fluorescens* ssp. indologenes T8 strain. The lowest lipolytic activity (5.3 mm) was detected in *P. vesicularis* T2 strain (Table 3).

DISCUSSION

Pseudomonas species have been reported as important decomposers of organic matter in soil, water and food products, but are also pathogens in plants, animals and humans (11). Water and soil are known as the primary sources of *Pseudomonas* sp. (12, 13). Hose nozzles and milking equipment can become colonized by *Pseudomonas*. Also, API 20 NE provided good identification of dairy *Pseudomonas* isolates to the species level (14, 15). In the present study, 15 *Pseudomonas* strains isolated from raw milk, identified by using API 20 NE and good identification was obtained.

Antibiotic resistance is an accepted concern for the management of disease in humans, animals and plants. The intense research efforts to elucidate mechanisms of resistance have focused on genes derived from a narrow range of environments (6). The *Pseudomonas* genus corresponds to a diverse and ecologically significant group of bacteria that are found in natural environments. Such a universal distribution can be associated with the capacity of *Pseudomonas* species to adapt to various environmental conditions and degrade a wide range of substrates (16). Most of the known resistance determinants have been discovered in clinical and veterinary bacterial isolates, whereas other environmental reservoirs of antibiotic resistance are not well characterized (6). It is not known whether antibiotic resistance genes move readily from environmental reservoirs to clinical settings, but future work should consider the potential contributions of soil bacteria to the problem of antibiotic resistance. Multiple drug resistant (MDR) bacteria in processed foods are potent biological hazards as there are possibilities for resistance genes to be spread to human beings via food (17). Antibiogram of the all the investigated isolates revealed that, of the seven antibiotics tested, almost all of the isolates was resistant to ampicillin and cefuroxime. Results are not suggestive of the trait of MDR in the isolate.

Pseudomonas spp. are the most important group of Psychrotrophes associated with spoilage, they grow rapidly at refrigeration temperatures and often dominate the microbial population Also, some *Pseudomonas* spp. have been reported to survive during heat treatment used in pasteurization of milk (18). It can be seen in previous studies that *Pseudomonas*, particularly *P. fluorescens*, was frequently isolated from refrigerated raw milk and associated with proteolysis and lipolysis (2, 19, 20). Extra cellular proteinases and lipases from psychrotrophic *Pseudomonas* are recognized as the primary microbial spoilage enzymes of dairy products (21, 22).

Craven and Macauley reported that 5% total of 26 *Pseudomonas* strains showed proteolytic activities (15 mm, zone diameter) in SMA (23). Same researchers revealed that 10% of *P. fluorescens* strains isolated from milk had proteolytic activity with 15-17 mm zone diameters (24).

In a previously performed study (25) the microflora of 19 samples of refrigerated bulk tank milk were examined and they found that the number of *Pseudomonas* spp. were increased significantly in milk stored at 2°, 4° and 7°C whilst other psychrotrophic flora decreased. Al-Ashmawy et al., found that lipolytic properties of the isolated *Pseudomonas* spp from table butter proved that all tested five *P. fragi* and three *P. fluorescens* strains produced lipolytic activity (26).

In this study we determined antibiotics susceptibility of *Pseudomonas* strains. Also we only assessed ability to produce protease and lipase enzymes on SMA and TA media to predict spoilage potential of strains. The use of microbial enzymes in food, pharmaceutical, textile, paper, leather, and other industries are numerous and are increasing rapidly. Therefore, determination and purification of microbial enzymes are very important for industrial applications.

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