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ARAŞTIRMA MAKALESİ **RESEARCH PAPER**

The Determination of Antibiotic Resistance and Biofilm Properties in *Pseudomonas aeruginosa* Isolates from Raw Milk Samples

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Abstract: In this study, it was aimed to investigate the antibiotic resistance profiles and biofilm formation properties of *Pseudomonas aeruginosa* isolates isolated from raw milks. A total number of 300 raw milk samples were collected from several dairy plants and vendors in the provinces of Aydın (n=100), İzmir (n=100) and Muğla (n=100) in Turkey. The conventional methods were used for the isolation of suspected *Pseudomonas* spp. from raw milk samples. A total of 63 suspected *Pseudomonas* spp. were isolated and these isolates were identified as being *P. aeruginosa* by PCR using *PA-SS* primers targeted to 16SrDNA gene. According to PCR results, 24 isolates were identified as *P. aeruginosa*. The antibiotic resistances of *P. aeruginosa* against 7 antibiotics were determined by Kirby-Bauer Disc Diffusion method. The antibiotic resistance rates of the isolates among enrofloxacine, ceftriaxone, ciprofloxacin, meropenem, colistin, gentamycin, and azithromycin were found as 20.8%, 75.0%, 4.2%, 33.3%, 8.3%, 12.5 % and 91.7%, respectively. When examined according to multidrug resistances, it was determined that 11 isolates (45.8%) were resistant to more than three antibiotic groups and were evaluated as multi-resistant. The biofilm formations of the isolates were investigated *in vitro* with Congo Red Agar (CRA) Method. The biofilm formation was determined at 9 isolates as the ratio of 37.5% with CRA method. The biofilm formation and multidrug resistance rates were found as high in the raw milk isolates of *P. aeruginosa*. In conclusion, the raw milk had considered to be a potential public health problem for *P. aeruginosa* and the widespread studies thought to be performed for the lightening of the biofilm related antibiotic resistances.

Keywords: Antibiotic resistance, biofilm, *Pseudomonas aeruginosa*, raw milk.

Çiğ Süt Örneklerinden İzole Edilen *Pseudomonas aeruginosa* İzolatlarında Antibiyotik Direnci ve Biyofilm Özelliklerinin Belirlenmesi

Öz: Bu çalışmada çiğ sütlerden izole edilen *Pseudomonas aeruginosa* izolatlarının antibiyotik dirençlilik profillerinin ve biyofilm oluşturma özelliklerinin araştırılması amaçlandı. Bu amaçla Aydın (n=100), İzmir (n=100) ve Muğla (n=100) illerindeki çeşitli süt işletme ve satıcılarından toplam 300 adet çiğ süt örneği toplandı. *Pseudomonas* spp. izolasyonu için geleneksel yöntemler kullanıldı. Çiğ süt örneklerinden toplam 63 şüpheli *Pseudomonas* spp. izole edildi ve bu izolatların *P. aeruginosa* yönünden identifikasyonu *PA-SS* primerleri kullanılarak 16S rDNA genini hedef alan PCR ile gerçekleştirildi. PCR sonuçlarına göre 24 izolat *P. aeruginosa* olarak tanımlandı. *P. aeruginosa* izolatlarının 7 antibiyotiğe karşı antibiyotik duyarlılıkları Kirby-Bauer Disk Difüzyon yöntemi ile belirlendi. İzolatların enrofloksasin, seftriakson, siprofloksasin, meropenem, kolistin, gentamisin ve azitromisin antibiyotiklerine direnç oranları sırasıyla %20,8, %75,0, %4,2, %33,3, %8,3, %12,5 ve %91,7 olarak bulundu. Çoklu antibiyotik dirençlerine göre

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incelendiğinde 11 izolatın (%45,8) üç'ten fazla antibiyotik grubuna dirençli olduğu belirlendi ve çoklu dirençli olarak değerlendirildi. İzolatların biyofilm oluşumları *in vitro* olarak Congo Red Agar (CRA) yöntemi ile araştırıldı. Biyofilm oluşum oranları CRA yöntemi ile 9 izolatta % 37,5 (9/24) olarak belirlendi. *P. aeruginosa'nın* çiğ süt izolatlarında biyofilm oluşumu ve çoklu antibiyotik direnç oranları yüksek bulunmuştur. Sonuç olarak çiğ süt örneklerinin *P. aeruginosa* için potansiyel halk sağlığı sorunu olduğu belirlendi ve bu durum ile biyofilm kaynaklı antibiyotik dirençliliklerinin aydınlatılması için daha geniş çaplı çalışmalar yapılması gerektiği düşünülmüştür.

Anahtar kelimeler: Antibiyotik direnci, biyofilm, çiğ süt, *Pseudomonas aeruginosa*.

INTRODUCTION

Charles-Emmanuel Sedillot was the first to characterize *Pseudomonas* in 1850. According to their rRNA homology, bacteria in the genus *Pseudomonas* are divided into five primary species clusters. *Pseudomonas aeruginosa* belongs to Group I's fluorescence subgroup (Brooks et al., 2007). In many animal species, *P. aeruginosa* causes opportunistic infections. Due to its opportunistic nature, *P. aeruginosa* thrives in the feces and skin of healthy animals, and any predisposing circumstances that depress the immune system of animals or disrupt the natural flora cause infection. *P. aeruginosa* pathogenicity is influenced by a variety of virulence factors. Flagella, fimbria (pilus), and lipopolysaccharide, polysaccharide capsule are among these virulence factors. The toxin and enzyme virulence factors of bacteria are phospholipase C (hemolysin), alginate-biofilm, siderophores (pyoverdine, pyocyanin, pyochelin), elastase (LasB and LasA), protease, leukocidin, exotoxin A, exotoxins S and T. Many of these virulence factors must coexist for bacteria to cause disease (Moore & Flaws, 2011).

Because of developing resistance to several medications, *P. aeruginosa* infections are extremely difficult to treat. Broad-spectrum penicillins, third-generation cephalosporins, carbapenems, monobactams, aminoglycosides, fluoroquinolones, and polymyxins are the most often used and therapeutically effective antibiotics for *P. aeruginosa* infections. Combinations of antibiotics are used in the treatment of infections in addition to the early and appropriate use of a single antibiotic. In general, combining aminoglycosides with beta-lactams, third-generation cephalosporins, monobactams, or carbapenems increases the likelihood of a successful treatment (Pitt & Simpson, 2006).

Biofilm generation is one of the most common reasons for *P. aeruginosa* treatment failure. Exopolysaccharide (EPS) in the biofilm structure is thought to have a crucial role in the bacterium's capacity to survive, and when EPS is eliminated from the biofilm under experimental settings, the bacteria become more susceptible to antimicrobial treatments (Watnick & Kolter, 2000). There's evidence that during attachment, the first stage of biofilm development, the levels of several genes rise. The microcolonies develop into biocide-resistant exopolysaccharide-coated communities after attachment. Many infectious diseases can be treated with effective antibiotic treatments. However, in cases where bacterial biofilms are mostly involved, this approach cannot provide an effective solution. Biofilms are thought to have this resistance through multiple mechanisms. The antimicrobial agent does not pass through all layers of the biofilm. It is known that polymeric substances in the biofilm matrix complicate the diffusion of antibiotics. This means that they never reach a sufficient concentration of antibiotics. At least some of the cells in the biofilm are nutrient deficient and therefore have to enter a slow growth phase. Slow-growing or non-growing cells are not susceptible to many antimicrobial agents and many can survive. There is an exchange of resistance genes between bacteria in the biofilm (Çiftçi et al., 2005).

In a literature, there are few informations about biofilm related antibiotic resistances in raw milk originated *P. aeruginosa*. In this study, it was aimed to determine the presence of biofilms and antibiotic resistances in *P. aeruginosa* isolates from raw milks.

MATERIAL AND METHOD

Sample collection: A total number of 300 raw milk samples were collected from several dairy plants and vendors in the provinces of Aydın (n=100), İzmir (n=100), and Muğla (n=100) in Turkey. The samples were transported to the laboratory in steril bags with cold conditions.

Isolation and identification of Pseudomonas aeruginosa: 10 mL of raw milk samples were added to 90 mL of 0.1% mL peptone water under aseptic conditions and that were homogenized with stomacher. Then 10^{-2} dilutions were prepared and 0.1 mL from this dilution were plated onto *Pseudomonas* Cephaloridine-Fucidin-Cetrimide (CFC) agar (Oxoid, SR103) by spread plate method. The plates were incubated 24-48 h at 30°C. The colonies which grew in *Pseudomonas* CFC agar and oxidase test positivity (oxidase paper, Merck 13300) were determined as suspected to be *P. aeruginosa*. (Mickova et al., 1989; Küplülü et al., 2003).

The suspected isolates were identified as being *P. aeruginosa* by PCR (Table 1). The pair PA-SS-F and PA-SS-R was designed to amplify only *P. aeruginosa*. These primers targeted species-specific signature sequences in 16S

rDNA variable regions 2 and 8 (V2 and V8), respectively (Spilker et al., 2004). Based on the 16S rDNA sequences, PA-SS region was designed simple, rapid, and accurate PCR assays that allow the differentiation of *P. aeruginosa* from other *Pseudomonas* species. Because of this reason, PA-SS oligonucleotide primers were used in PCR. The 956 bp band after PCR was considered positive for *P. aeruginosa*.

Determination of antibiotic resistance profiles: Kirby-Bauer Disk Diffusion method was used to determine the antibiotic susceptibility of the isolates (CLSI, 2018). For this purpose, antibiotic discs of azithromycin (30 µg), gentamicin (10 µg), colistin (10 µg), meropenem (10 µg), ciprofloxacin (5 µg), ceftriaxone (30 µg), enrofloxacin (5 µg) were used. At the end of the incubation, the inhibition zone diameters were measured and the antibiotic susceptibilities of the isolates were evaluated according to the values reported in EUCAST (2018) and CLSI (2018).

Determination of in vitro biofilm production: Biofilm formation of the isolates was investigated *in vitro* using Congo Red Agar (CRA) method. CRA method was carried out with the method reported (Atshan et al., 2012). For this aim, CRA was prepared with 10 grams of agar, 50 grams of glucose, 37 grams of Brain-Heart Infusion Broth, and 0.8 grams of Congo red dye in 1000 mL of distilled water. By taking a single colony from the purely breeding colonies in TSA, it was transferred to CRA. The media were incubated at 37°C for 24-48 hours under aerobic conditions. At the end of the incubation period, the color changes in the colonies were evaluated. Isolates with black-gray colony formation on CRA were evaluated as positive for biofilm production, and pink-red colonies were evaluated as negative.

RESULTS

Isolation and identification: The suspected *Pseudomonas* spp. were isolated from 63 (21%) of 300 raw milk samples. Based on the distribution of 63 suspected *Pseudomonas aeruginosa* raw milk samples, 17 (27%) were isolated from Aydın, 21 (33.3%) from İzmir and 25 (39.7%) from Muğla. The twenty-four (38.1%) of 63 suspected isolates were determined as *P. aeruginosa* after PCR. Of the 24 *P. aeruginosa* isolates, 5 (20.8%) isolates were from Aydın, 8 (33.3%) isolates were from İzmir and 11 (45.8%) isolates were from Muğla originated raw milk samples.

Table 1. Oligonucleotide primer sequences used for identification of *Pseudomonas aeruginosa*.

Primers		Oligonucleotide sequences	Expected amplicon sizes (bp)	Reference
PA-SS	F	GGGGGATCTTCGGACCTCA	956	Spilker et al., (2004)
	R	TCCTTAGAGTGCCCACCCG		

Table 2. Antibiotic resistance profiles of the isolates.

	Antibiotics						
	ATM	GEN	C	MRP	CIP	CTX	ENR
	n (%)	n (%)	n (%)	n (%)	n (%)	n (%)	n (%)
R	22 (91.7)	3 (12.5)	2 (8.3)	8 (33.3)	1 (4.2)	18 (75.0)	5 (20.8)
I	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
S	2 (8.3)	21 (87.5)	22 (91.7)	16 (66.7)	23 (95.8)	6 (25.0)	19 (79.2)

*S: sensitive, I: intermediate resistant, R: resistant, ATM: azithromycin, GEN: gentamicin, C: colistin, MRP: meropenem, CIP: ciprofloxacin, CTX: ceftriaxone, ENR: enrofloxacin.

Determination of antibiotic resistance profiles: By Kirby-Bauer Disc Diffusion Method applied to *P. aeruginosa* isolates, resistances of 20.8% for enrofloxacin, 75.0% for ceftriaxone, 4.2% for ciprofloxacin, 33.3% for meropenem, 8.3% for colistin, 12.5% for gentamicin, and 91.7% for azithromycin were determined (Table 2). When examined according to multidrugresistances, it was determined that 11 isolates (45.8%) were resistant to more than three antibiotic groups and were evaluated as multidrug resistant (Table 3).

Table 3. The multidrug resistance profiles of the isolates.

The number of antibiotics (n)	*P. aeruginosa*	
	n	%
4	3	12.5
3	8	33.3
2	18	75.0

Determination of in vitro biofilm production: The biofilm formation status of *P. aeruginosa* isolates (n=24) was investigated by CRA method, and it was determined that nine isolates (37.5%) had biofilm activity.

DISCUSSION AND CONCLUSION

P. aeruginosa has been known as a common opportunistic pathogen that reasons cause nosocomial infections in patients with immunocompromise. *P. aeruginosa* infection in many patients is a public health problem. In the study that was aimed to prevalence the exotoxin genes encoded type Ш secretion system and pattern of antimicrobial susceptibility of *P. aeruginosa* isolated from clinical and raw milk specimens, 6 (5%) of 120 raw milk samples were found positive for *P. aeruginosa* (Jarjees, 2020). This isolation prevalence is lower than the isolation prevalence of this study that was

as 24 isolates from total of 300 raw milk samples (8%). A total of 14 raw milk samples were collected from several dairy plants in Ankara, Turkey for the isolation of *Pseudomonas* spp. and a total of 55 isolates were isolated by using Cetrimide Agar. *Pseudomonas* spp. accounted for 85.5% of the total isolates and among these isolates *P. aeruginosa* accounted as 34.6% (Akoğlu et al., 2012), and that is too higher than our isolation prevalence. The 120 retail samples of raw milk, Kareish cheese and butter were carried out to determine the presence of *Pseudomonas* spp. and a total of 25 (83.3%) *P. aeruginosa* strains identified from 30 raw milk samples (Sadek et al., 2006) as nearly 10 times higher than the prevalence of this study. The 50 raw milk samples from various villages in the provinces of Kayseri and Niğde in Turkey were collected and a total of 1 (2%) *P. aeruginosa* strain were isolated (Taş et al., 2013) as 4 times lower than the prevalence of our study. It was aimed to detect the prevalence of *P. aeruginosa* in milk (n=125) samples which were collected from local vendors, private dairy farms in and around Tirupati and *P. aeruginosa* was isolated from total of 19 (15.2%) milk samples (Swetha et al., 2017) and this prevalence is nearly 2 times higher than the prevalence in our study. The collected raw milk samples (n=75) from various local milk collection centers in different parts of Kanchipuram district, South India to enumerate and examined to identify bacteria with proteolytic and lipolytic activity. *P. aeruginosa* was isolated from total of 7 (9.3%) raw milk samples (Parkash et al., 2007) as nearly similar to the prevalence in this study. A total of 100 random samples of raw milk from different markets in Port-Said city were collected for isolation and identification of *Pseudomonas* species. Six isolates of *Pseudomonas* species were isolated from 100 raw milk samples and all the 6 (6%) isolates were identified as *P. aeruginosa* (Mohammed et al., 2015). This prevalence is lower than the prevalence in our study. It was reported that *P. aeruginosa* from 11 (5.5%) raw milk samples was isolated from total of 200 raw milk samples (Uraz and Çıtak, 1998) and this prevalence is lower than the prevalence we found in our study. A total of 200 samples of raw and pasteurized milk (100 of each) were collected from supermarkets in Qaluobia Governorate and examined for prevalence and characterization of *P. aeruginosa*. The incidence of *P. aeruginosa* isolated from raw milk was 40% (El-Roos et al., 2013) and this prevalence is 5 times higher the prevalence of our study.

P. aeruginosa is considered as one of the major pathogens of fleece rot in sheep, mastitis and abortion in cattle, metritis and corneal ulcer in horses, otitis externa in dogs, hemorrhagic pneumonia in mink, embryo death in poultry, necrotic stomatitis in cage snakes, botryomycosis, septicemia, animal urinary system infection, and wound infection. Anti-pseudomonal penicillins, aminoglycosides, cefepime, ceftazidime, ciprofloxacin, meropenem and imipenem are antibiotics of choice for *P. aeruginosa* infections. *P. aeruginosa* infections are very difficult to treat because the bacteria can develop resistance to many antibiotics. Due to the importance of multidrug resistance in *P. aeruginosa* strains, antimicrobial susceptibility tests are considered important. In this study, Kirby-Bauer Disk Diffusion Method and antibiograms were applied on *P. aeruginosa* isolates. Antibiotic resistance rates of enrofloxacine, ceftriaxone, ciprofloxacin, meropenem, colistin, gentamycin and azithromycin isolates were determined as 20.8%, 75.0%, 4.2%, 33.3%, 8.3%, 12.5% and 91.7%, respectively. In a study conducted in 2012 included in the reports of the National Antimicrobial Resistance Surveillance System (UAMDSS), the antibiotic susceptibility status of 1209 *P. aeruginosa* strains was determined.

In this study, it was reported that the highest resistance was against azithromycin (91.7%), the second highest resistance was against ceftriaxone (75.0%) and the lowest resistance was against ciprofloxacin (4.2%). Resistance to meropenem was 33.3%, enrofloxacin resistance rate was 20.8%, gentamicin resistance rate was 12.5% and colistin resistance rate was 8.3%. As a result of the comparison of the 2012 data published by UAMDSS and EARS-Net (European Antimicrobial Resistance Surveillance Network), which includes 30 countries, it was seen that the resistance against piperacillin / tazobactam, ceftazidime and fluoroquinolones in our country was above the European average. According to this report, the rate of antibiotic resistance in strains acquired in Turkey appears to be higher than the European average.

It is known that the vegetative / planktonic forms of microorganisms as well as biofilm structures play an important role in the pathogenesis of many infectious diseases including *P. aeruginosa*. With the increase in the prevalence of biofilm infections, research on the control and prevention of biofilm formation has gained momentum. Today, many different *in vitro* and *in vivo* methods based on biofilm infection in experimental animals are used to detect biofilm formation with the help of developing technology. CRA, Christensen and microplate methods stand out as frequently used methods in detecting biofilm formation. Yassein et al., (1995) detected the presence of biofilm in 40% of 50 *P. aeruginosa* strains isolated from clinical samples. Delissalde and Amabile-Cuevas, (2004) reported biofilm formation in 18% of 162 *P. aeruginosa* isolates, which are hospital infection agents. Moskowitz et al., (2005) reported positive biofilm formation in 73% of 96 strains isolated from various services and clinics. According to Coban et al., (2009) reported that 33.3% of 60 *P. aeruginosa* strains isolated from patients with cystic fibrosis had biofilm

positivity. Biofilm positivity was found by Yıldırım et al., (2008) and Çelik et al., (2007) in 45% and 48% of the isolates, respectively. Milivojevic et al., (2018) reported that 93% of animal and 96% of human isolates formed biofilms *in vitro* in their study on a total of 202 *P. aeruginosa* isolates from 121 animals and 81 human origins. Olejnickova et al., (2014) found that the rate of biofilm production in isolates originating from human catheters varied between 80-88% and reported that studies on animal isolates were very limited. The rate of biofilm formation in *P. aeruginosa* isolates from canine otitis was reported as 40% and these isolates did not respond to treatment (Pye et al., 2013). In our study, biofilm formation status of isolates was investigated by CRA method. It was determined that 9 (37.5%) isolates according to the CRA method. These results indicated that the biofilm formation were high in raw milk isolates of *P. aeruginosa* strains.

In conclusion, biofilm production and multidrugresistance were found to be high in clinical isolate *P. aeruginosa* strains. At the genetic level, it was determined that the examined genes were not solely responsible for biofilm formation and related antibiotic resistance. This suggests that different and more genetic and phenotypic parameters may be responsible for biofilm-induced antibiotic resistance. These results indicated that the raw milk had considered to be a potential public health problem for *P. aeruginosa*. The widespread and advanced studies thought to be performed for the lightening of this situation and determining the relevant parameters and to elucidate the mechanisms of biofilm-associated antibiotic resistance.

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