Etlik Vet Mikrobiyol Derg, 2022; **33 (2)**
**doi:** https://doi.org/10.35864/evmd.1166225

**Original Article**
Özgün Araştırma

# Investigation of the antibiotic resistances, biofilm formation, and genotypes of *Pseudomonas aeruginosa* isolates from Turkey and Romania

**Merve Gizem Sezener[1], Volkan Enes Ergüden[2], Arzu Fındık[3], Serhan Akgöz[4], Timur Gülhan[5], Oana–Alexandra Moţco (Ciocan)[6], Alper Çiftci[7]***

*[1,2,3,4,5,7] Department of Veterinary Microbiology, Faculty of Veterinary Medicine, University of Ondokuz Mayis, Atakum, Samsun, Turkey*
*[6] Dialab Solutions, Str. Albac 11, Bucureşti, Romania*

**Geliş Tarihi** / Received: 24.08.2022, **Kabul Tarihi** / Accepted:10.10.2022

**Abstract:** *Pseudomonas aeruginosa* is frequently isolated from various disease and nosocomial infections. It is clinically important because of its multiple antibiotic resistance and biofilm production. The aims of this study were to determine the production of biofilm and to make antibiotyping and genotyping of *P. aeruginosa* strains isolated from bronchial aspirate samples of dogs with pneumonia in Turkey and Romania. For this purpose, biofilm properties of 10 Turkish and 10 Romanian isolates were determined by Congo Red Agar Method. The resistance profiles of the isolates were determined by Kirby-Bauer Disk Diffusion Method. The antibiotyping was performed according to the resistance profiles of the isolates. Genotypical similarities were determined by RAPD-PCR. In terms of biofilm production, all of the isolates examined in this study were found to be positive. In consequence of the antibiograms, the resistance rates against enrofloxacin, ceftriaxone, amoxicillin + clavulanic acid, ciprofloxacin, meropenem, gentamicin and azithromycin were stated as 30%, 70%, 100%, 40%, 30%, 10%, 100% for Turkish isolates and 30%, 60%, 100%, 30%, 10%, 10%, 100% for Romanian isolates, respectively. As a result of antibiotyping, similarities of the isolates from Turkey and Romania were determined to be between 86-100%. Phylogenotypical similarities of the isolates from Turkey and Romania were determined to be between 47-96%. It was concluded that antibiotic resistances of isolates were high and resistance against antibiotics used in the treatment could be formed due to biofilm production.

**Keywords:** Antibiotyping, Biofilm, Genotyping, *Pseudomonas aeruginosa*.

## Türkiye ve Romanya kökenli *Pseudomonas aeruginosa* izolatlarının antibiyotik dirençlerinin, biyofilm oluşumu ve genotiplerinin araştırılması

**Özet:** *Pseudomonas aeruginosa* çeşitli hastalık olgularından ve hastane infeksiyonlarından sıklıkla izole edilmektedir. Çoklu antibiyotik direnç özelliği ve biyofilm üretimine sahip olması nedeniyle klinik açıdan önem arz etmektedir. Bu çalışmada Türkiye ve Romanya'da pnömoni bulguları gösteren köpeklerden alınan bronşiyal aspirat örneklerinden izole edilen *P. aeruginosa* suşlarının biyofilm üretiminin belirlenerek antibiyotiplendirme ve genotiplendirilmelerinin yapılması amaçlanmıştır. Bu amaçla Türkiye ve Romanya izolatı 10'ar adet *P. aeruginosa* izolatının biyofilm özelliği Kongo Red Agar yöntemi ile belirlendi. İzolatların antibiyotik direnç durumları Kirby-Bauer Disk Difüzyon Metodu ile belirlendi ve direnç profiline bağlı olarak antibiyotiplendirme yapıldı. Genotipik yakınlıklar RAPD-PCR ile belirlendi. Çalışmada incelenen tüm izolatların biyofilm üretimi yönünden pozitif olduğu saptandı. Antibiyogram sonucunda Türkiye izolatlarının enrofloksasin, seftriakson, amoksisilin + klavulanik asit, siprofloksasin, meropenem, gentamisin ve azitromisin içeren disklerle karşı direnç durumları sırasıyla %30, %70, %100, %40, %30, %10, %100 ve Romanya izolatlarının da sırasıyla %30, %60, %100, %30, %10, %10 ve %100 olduğu belirlendi. Antibiyotiplendirme sonucunda, Türkiye ve Romanya izolatlarının %86-100 arasında benzerlik gösterdiği belirlendi. Filogenetik değerlendirme ile Türkiye ve Romanya izolatlarının filogenotipik benzerliklerinin %47-96 arasında olduğu belirlendi. Sonuç olarak, izolatların antibiyotik direncinin yüksek olduğu ve biyofilm üretimine bağlı olarak tedavide kullanılan antibiyotiklere karşı direnç şekillenmiş olabileceği kanısına varıldı.

**Anahtar kelimeler:** Antibiyotiplendirme, Biyofilm, Genotiplendirme, *Pseudomonas aeruginosa.*

***Yazışma adresi / Correspondence:*** *Prof.Dr. Alper Çiftci, University of Ondokuz Mayıs, Faculty of Veterinary Medicine, Department of Veterinary Microbiology, Atakum, Samsun, Turkey e-mail: aciftci@omu.edu.tr*

***ORCID IDs of the authors:*** *[1]0000-0003-0487-7515 • [2]0000-0003-2215-2868 • [3]0000-0002-9123-6160 • [4]0000-0002-5130-7120 • [5]0000-0003-4798-1427 • [6]0000-0002-9342-2637 • [7]000-0001-8370-8677*

## Introduction

*Pseudomonas* spp. is an important group among opportunistic Gram negative bacteria that cause nosocomial infections, especially in immunosuppressive patients. As the frequency of broad spectrum antibiotic usage increases, the number of bacteria with multiple antibiotic resistance is increasing. This resistance has become particularly prominent in Gram-negative bacteria (Verbist, 1991).

*Pseudomonas* spp. is a Gram-negative, non-spore forming, aerobic rod-shaped bacterium. They grow more easily in humid environments. *Pseudomonas* containing a large number of species are classified according to their pigmentation and metabolism. *Pseudomonas aeruginosa* is the most common species isolated from disease cases in the genus (Erdem, 1999). *P. aeruginosa* produces pigments such as pyoverdin, and pyocyanin (Wyngaarden et al., 1992; Bilgehan, 1994). Temporary colonization of the *Pseudomonas* is seen on perineum, outer ear canal, armpit and lower genital tract in healthy individuals. In addition, this colonization can be turn into infection in immunocompromised individuals or individuals whose natural microbiota is disrupted due to long-term usage of broad-spectrum antibiotics (Bilgehan,1994; Pollack, 1995).

Biofilm is a complex organization where bacteria live in a harmonious and structural integrity by adhering to a surface (Kumar et al., 2011). Microorganisms form a community which has been defined as microecosystem for sustaining their vital activities and protecting themselves from environmental conditions that have negative effects on their lives (Marcinkiewicz et al., 2013). Biofilm was first identified by Antonie van Leeuwenhoek in the 17th century. The researcher examined the swab taken from his own tooth plaque and detected the formation of microbial clusters under the microscope (Donlan and Costerton, 2002).

Biofilm structure consists of organic exopolysaccharides produced by microorganisms themselves. Microorganisms create biofilms after attaching them to a living or inanimate surface, and this structure is advantageous in terms of escape from host defense and resistance to antibiotics. Also biofilm formation in microorganisms cause to acquisition of resistance against disinfectants, pH change and dryness (Høiby et al., 2011). Biofilms pose a problem in environmental and industrial context and can also cause clinical problems (Costerton et al.,1999). The biofilm-forming bacteria are more resistant against antibiotics than vegetative bacteria (Ceri et al., 1999; Wand et al., 2012). Since many antibiotics targets rapidly dividing bacteria, antibiotics are not effective on bacteria present in the deep layers of the biofilm that exhibits reduced metabolic activity and division rates. Therefore, effects of the antibiotics on the bacteria which is close to the surface in the biofilm are bactericidal, however, the bacteria remaining deep parts inside the biofilm survives and creates an environment for re-development (Çiftci et al., 2005).

There are so many bacterial typing techniques are currently available for discriminating the bacterial strains. These techniques vary with respect to the effort required, cost, and reliability. None of these techniques is optimal for all investigations. Traditional typing systems for discriminating between bacteria from a single species have been based on phenotype. Serotyping, biotyping, phage-typing, or antibiogram (susceptibility to one or more antibiotics) are some examples of phenotype based typing. *In vitro* antimicrobial susceptibility patterns are still used in epidemiological investigations since antibiograms are rapidly delivered to infection control specialists without the need for additional investigations. In addition, new methods based on genetic variations of bacteria were currently in use. RAPD-PCR is a widely used method for genotyping various bacterial species. Comparison of RAPD-PCR and other genotyping results with antibiotic resistance profiles may be important in terms of showing the specific risk of resistance to treatment in infectious diseases, by showing whether some specific genotypes are similar to those of multiple resistance strains.

The aims of this study were to determine the production of biofilm and to perform antibiotyping and genotyping of *P. aeruginosa* strains isolated from bronchial aspirate samples of dogs with pneumonia in Turkey and Romania.

## Materials and Methods

### Bacterial strains

Total of 20 *P. aeruginosa* isolates including 10 Turkish- and 10 Romanian-originated isolates in a collection of Veterinary Microbiology Department of the University of Ondokuz Mayıs, Faculty of Veterinary Medicine were investigated in a study. All strains have been known to be isolated from bronchial aspirate samples taken from dogs showing pneumonia symptoms. The strains were inoculated on 7% sheep Blood Agar (Sigma, 70133) and incubated for 24 h at 37°C. After incubation period, colonies

were checked for Gram staining and oxidase characteristics.

### Determination of biofilm formation

Congo Red Agar Method was used to determine biofilm formation. For this purpose, Brain-Heart Infusion agar (Merck, 70138) containing 3% sucrose (Sigma, 1076535000) and 0.08% Congo red (Sigma, 75768) was prepared. Suspected colonies were inoculated on prepared agar at 37°C for 48 h. Black colony formation was evaluated as positive for biofilm and pink colonies as negative (Atshan et al., 2012).

### Antimicrobial susceptibility testing and antibiotyping

The Kirby-Bauer Disc Diffusion Method was used to determine the resistance of the isolates against antibiotics. For this purpose, 100 µl of bacterial suspensions adjusted to Mc Farland 0.5 were taken and inoculated on Mueller-Hinton Agar. The antibiotic discs containing enrofloxacin (5 µg) (Thermo Fisher, CT0639B), ceftriaxone (30 µg) (Thermo Fisher, CT0417B), amoxicillin + clavulanic acid (20+10 µg) (Thermo Fisher, CT0223B), ciprofloxacin (5 µg) (Thermo Fisher, CT0425B), meropenem (10 µg) (Thermo Fisher, CT0774B), gentamicin (10µg) (Thermo Fisher, CT0024B), and azithromycin (15 µg) (Thermo Fisher, CT0906B) were placed on agar. After incubation at 37°C for 24 h, zone diameters were evaluated according to CLSI (2018).

The antibiotyping was performed according to the resistance profiles of the strains by means of the Unweighted Pair Group Method using arithmetic averages (UPGMA) cluster analysis, and the dendrogram was created for evaluation of relatedness between the strains.

### Genotyping

RADP-PCR was performed for phylogenetic typing. ERIC-2 oligonucleotide primer was used to determine RAPD-PCR patterns of the strains. The amplification was performed by modifying the method reported by Versalovic and Lupski (2002). Dendrograms were generated from RAPD-PCR of strains using UPGMA.

## Results

### Determination of biofilm formation

According to the Congo red agar method, all colonies which found to be black colored were evaluated as biofilm positive.

### Antimicrobial susceptibility testing and antibiotyping

According to the antibiogram results, the antibiotic resistance states of Turkish isolates found to be 30%, 70%, 100%, 40%, 30%, 10%, and 100% against enrofloxacin, ceftriaxone, amoxicillin+clavulanic acid, ciprofloxacin, meropenem, gentamicin, and azithromycin, respectively. In the Romanian isolates, resistance rates were found to be 30%, 60%,100%, 30%, 10%, 10%, and 100% against enrofloxacin, ceftriaxone, amoxicillin + clavulanic acid, ciprofloxacin, meropenem, gentamicin, and azithromycin, respectively. Six Turkish and five Romanian originated isolates were found to show multidrug resistance.

According to the results of dendrogram drawn by the method of UPGMA, the similarities of Turkish and Romanian isolates were found to be 80-100% and 86-100%, respectively. In a total, the strains had a similarity of 86-100% (Figure 1).

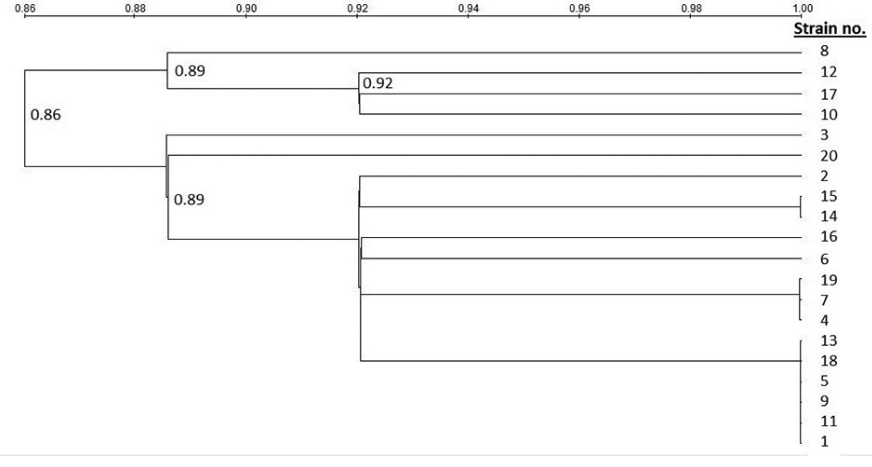


**Figure 1.** The antibiotyping results of *P.aeruginosa* strains. 1-10: Turkish originated strains; 11-20: Romanian originated strains.

### Genotyping

As a result of the dendrogram, the phylogenotypic similarity rates between Turkish and Romanian strains were determined to be between 47-96% (Figure 2). Similarities were detected between 40-82% in Turkish originated isolates, while Romanian isolates were between 74-95%. One of the Turkish isolate could not be typed.

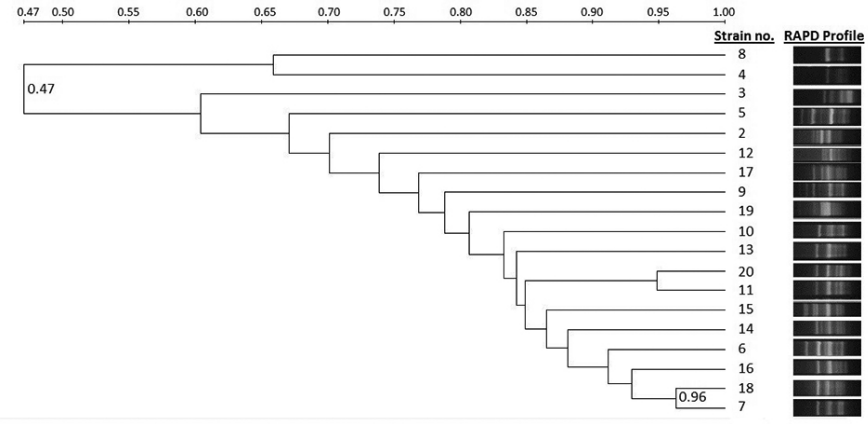


**Figure 2.** The phylogenotyping results of *P.aeruginosa* strains. 2-10: Turkish strains; 11-20: Romanian strains.

## Discussion

*Pseudomonas* spp. have a great importance among opportunistic Gram-negative bacteria that cause nosocomial (community-originated) infections in long-term hospitalized patients or immunocompromised individuals. The number of bacteria with multiple antibiotic resistance increases rapidly with the use of unconscious antibiotics and the frequency of the broad spectrum antibiotic usage in treatment. This kind of resistance has become extremely common, especially in Gram negative bacteria (Verbist, 1991). Due to the increased resistance to antibiotics in the treatment of infections caused by biofilm-forming strains of *P. aeruginosa*, the treatment of infections becomes very difficult (Jennings et al., 2015). In the study conducted by Manohar et al. (2018), 8 of 24 *P. aeruginosa* strains isolated from various clinical cases were found positive for biofilm production. Furthermore, in conclusion of the conducted antibiogram test 63% of the strains were found to be resistant to gentamicin, 71% to ciprofloxacin, and 50% to meropenem. In the study conducted by Sharma et al. (2015) in India, 47 *P. aeruginosa* strains were examined for biofilm production and antibiotic resistance. As a result of this study, 42 strains were found to have biofilm activity. They also found that resistance rates of the strains were 78.5% for gentamicin, 83.3% for tobramycin, 80.9% for amikacin and 73.8% for kanamycin. When the results of both studies were compared with the antibiotic susceptibility results of our study, it was thought that the rate of resistance to gentamicin was very high and this may be due to the high frequency of antibiotic usage in the treatment of infections. In France, Haenni et al. (2015)'s study revealed that 16 (35%) of 46 *P. aeruginosa* strains isolated from dogs had multiple antibiotic resistance. In our study, this situation were found to be 6/10 (60%) on isolates of Turkey originated, 5/10 (50%) of Romanian isolates, respectively. Multiple resistance mechanisms are mainly the synthesis of beta lactamases, reduction of bacterial outer membrane permeability and efflux systems. If efflux systems are activated by chromosomal beta-lactamase enzymes, multiple drug resistance to many antibiotics may occur. This may happen during treatment and resistant strains may appear (Gür, 1999). Frequent and uncontrolled usage of antibiotics by clinicians increases the rate of emergence of resistance. Therefore, effective treatment protocols should be determined. For this purpose, identification of disease agents and antibiotic susceptibilities should be routinely determined (Köseoğlu Eser et al., 2005).

Several methods have been used in the epidemiological studies to classify bacterial isolates from phenotypic and genotypic perspective in the last two decades. The applicability of phenotypic methods including serotyping, phage typing and biotyping is limited, because of the difficulty of obtaining standard antiserum and phage reagents, the lack of protocol standardizations between laboratories, presence of too many non-typable strains and

being time-consuming (Nielsen et al., 2000; Wassenaar and Newell, 2000; Shi et al., 2002). In order to overcome these drawbacks, genotypic methods have been developed based on the determination of specific molecular properties of chromosomal or plasmidic DNA (Shi et al., 2002). Commonly used genotypic methods are Pulsed field gel electrophoresis, randomly amplified polymorphic DNA typing (RAPD) and restriction fragment length polymorphism (RFLP) (Nielsen et al., 2000; Ono et al., 2003). RAPD-PCR, a method designed to investigate the genetic differences of bacteria isolated from different sources, is based on various primers and reaction conditions and as a method it is easy to do and cost-effective for large number of isolates (Hilton et al., 2006). In study conducted by Sallman et al. (2018) in Iraq, they found that 61 *P. aeruginosa* strains isolated from different clinical cases showed changing similarity rates between 25-92% with RAPD-PCR. In a study conducted by Ozturk et al. (2010), isolated 43 *P. aeruginosa* strain could not be typed because of their genetic diversity. According to the results of antibiograms, the dendrogram drawn by the method of UPGMA, the similarities of Turkish and Romanian isolates were stated to be 80-100% and 86-100%, respectively. It has been found that Turkish and Romanian isolates had shown similarities between 86-100%. In RAPD-PCR, one of the Turkish strain didn't yield a band. As a result of the dendrogram, the phylogenotypic similarity between Turkey and Romania isolates were determined to be between 47-96%. Similarities of the strains were detected between 40-82% in Turkish isolates, while Romanian isolates were between 74-95%.

In conclusion, it was determined that the resistance profiles of *P. aeruginosa* strains isolated from respiratory infections in dogs differed in both countries and the resistance were high. Biofilm, formations have been determined to be produced by all strains and it is known that produced biofilm plays an important role in escape of host defense mechanisms and resistance to antibiotics. Therefore, resistance profiles must be determined in antimicrobial treatment against biofilm producing bacteria. It is also important to shed light on the mechanisms of biofilm resistance in the elimination of the infections, which leads to a significant increase in therapeutic costs as well as therapeutic problems. In order to prevent antibiotic resistance, appropriate antibiotics should be used in the treatment of infections. Human and veterinary drug monitoring programs should be effectively maintained. Also, the continuous trainings and incentive programs should be organized for the use of appropriate drugs, profiles of resistant bacteria should be determined, infection prevention strategies should be developed and R&D studies should be carried out for the development of new antimicrobial agents.

## References


Atshan SS, Shamsudin MN, Lung LT, Sekawi Z, Ghaznavi-Rad E, Pei CP. (2012). Comparative characterisation of genotypically different clones of MRSA in the production of biofilms. *J Biomed Biotechnol.* 2012, 417-427.

Bilgehan H. (1994). Klinik Mikrobiyoloji. 8. Basım, İzmir: Barış Yayınları Fakülteler Kitabevi, 139-145.

Ceri H, Olson ME, Stremick C, Read RR, Morck D, Buret A. (1999). The calgary biofilm device: new technology for rapid determination of antibiotic susceptibilities of bacterial biofilms. *J Clin Microbiol.* 6(37), 1771-1776.

Clinical and Laboratory Standards Institute (2018). Performance standards for antimicrobial susceptiblity testing: 26th informational supplement, M100-S26, Wayne, PA.

Costerton JW, Stewart PS, Greenberg EP. (1999). Bacterial Biofilms: A Common Cause of Persistent Infections. *Science*. 284, 1318-1322.

Çiftçi İH, Çetinkaya Z, Aktepe OC, Arslan F, Altındiş M. (2005). Klinik Örneklerden İzole Edilen *Pseudomonas aeruginosa* Suşlarının Antibiyotiklere Duyarlılıkları. *Türk Mikrobiyol Cem Derg.* 35, 98-102.

Donlan RM, Casterton JW. (2002). Biofilms: Survival mechanisms of clinically revelant microorganisms. *Clin Microbiol Rev.* 15(2), 167-193.

Erdem B. (1999). *Pseudomonaslar*. In: Ustaçelebi Ş, Mutlu G, İmir T (Eds). Temel ve Klinik Mikrobiyoloji. 10. Baskı. Ankara: Güneş Kitabevi, 551-558.

Gür D. (1999). Hastane infeksiyonu etkeni Gram negatif nonfermentatif basiller ve antibiyotiklere direnç sorunu. *Hast İnfeks Derg.* 3(1), 33-39.

Haenni M, Hocquet D, Ponsin C, Cholley P, Guyeux C, Madec JY, Bertrand X. (2015). Population structure and antimicrobial susceptibility of *Pseudomonas aeruginosa* from animal infections in France. *BMC Vet Res.* 11, 9.

Hilton AC, Mortiboy D, Banks JG, Penn CW. (2006). RAPD analysis of environmental, food and clinical isolates of *Campylobacter* spp. *FEMS Immunol Med Microbiol.* 18 (2), 119-124.

Høiby N, Ciofu O, Johansen HK. (2011). The clinical impact of bacterial biofilms. *Int J Oral Sci.* 3(2), 55-65.

Jennings LK, Storek KM, Ledvina HE, Coulon C, Marmont LS, Sadovskaya I, Secor PR, Tseng BS, Scian M, Filloux A, Wozniak DJ. (2015). Pel is a cationic exopolysaccharide that cross-links extracellular DNA in the *Pseudomonas aeruginosa* biofilm matrix. *Proc Natl Acad Sci.* 112, 11353-11358.

Köseoğlu EÖ, Kocagöz S, Ergin,A. (2005). Yoğun bakım ünitelerinde infeksiyon etkeni olan gram-negatif basillerin değerlendirilmesi. *İnfeks Derg.* 19(1), 75-80.

Kumar MA, Anandapandian KTK, Parthiban K. (2011). Production and characterization of exopolysaccharides (EPS) from biofilm forming marine bacterium. *Braz Arch Biol Technol.* 54(2), 259-265.

Manohar P, Shanthini T, Philip RA, Ramkumar S, Kale M, Ramesh N. (2018). Role of biofilm-specific antibiotic resistance genes PA0756-0757, PA5033 and PA2070 in *Pseudomonas aerugi-*

*nosa*. *bioRxiv* preprint first posted online Jun. 11, 2018. doi: http://dx.doi.org/10.1101/341826.

Marcinkiewicz J, Strus M, Pasich E. (2013). Antibiotic resistance: a "dark side" of biofilm associated chronic infections. *Pol Arch Med Wewn.* 123(6), 309-313.

Nielsen EM, Engberg J, Fussing V, Petersen L, Brogren CH. (2000). Evaluation of phenotypic and genotypic methods for subtyping *Campylobacter jejuni* isolates from humans, poultry, and cattle. *J Clin Microbiol.* 38(10), 3800-3810.

Ono K, Kurazono T, Niwa H, Itoh K. (2003). Comparison of three methods for epidemiological typing of *Campylobacter jejuni* and *C. coli*. *Curr Microbiol.* 47, 364-371.

Öztürk O, Öztürk C, Delialioğlu N, Emekdaş G. (2010). Çoklu Antibiyotik Dirençli Gram Negatif Bakterilerde Kolistin Duyarlılığının Belirlenmesi. *Mersin Univ Sağlık Bilim Derg.* 3(3), 15-20.

Pollack M. (1995). *Pseudomonas* Infections. In: Mandell GL, Bennett JE, Dolin R (eds). Principles and Practice of Infectious Diseases,4th edt, New York: Wiley Medical Publication, 665-671.

Sallman RS, Hussein SS, Ali MR. (2018). ERIC-PCR Typing, RAPD-PCR Fingerprinting and Quorum Sensing Gene Analysis of *Pseudomonas aeruginosa* Isolated from Different Clinical Sources. *Al-Mustansiriyah J Sci.* 29(2), 51-62.

Sharma I, Choudhury D. (2015). Detection of *PelA* Gene in *P. aeruginosa* from Clinical Samples Using Polymerase Chain Reaction with Reference to Biofilm Production In N.E India. *Medi Sci.* 4(10), 119-121.

Shi F, Chen YY, Wassenaar TM, Woods WH, Coloe PJ, Fry BN. (2002). Development and application of a new scheme for typing *Campylobacter jejuni* and *Campylobacter coli* by PCR-based Restriction Fragment Length Polymorphism Analysis. *J Clin Microbiol.* 40(5), 1791-1797.

Verbist L. (1991). Incidence of multi-resistance in gram negative bacterial isolates from intensive care units in Belgium: a surveillance study. *Scan J Infect Dis.* 78, 45.

Versalovic J, Lupski JR. (2002). Molecular detection and genotyping of pathogens: more accurate and rapid answers. *Trends Microbiol.* 10, 15-21.

Wand ME, Bock LJ, Turton JF, Nugent PG. (2012). *Acinetobacter baumannii* virulence is enhanced in *Galleria mellonella* following biofilm adaptation. *J Med Microbiol.* 61, 470-477.

Wassenaar TM, Newell DG. (2000). Genotyping of *Campylobacter* spp. *Appl Environ Microbiol.* 66(1), 1-9.

Wyngaarden SB. (1992). Cecil Textbook of Medicine. WB. Sauders Philadelpia Company, 1717-1721.