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Original Article
Özgün Araştırma

Antibiotic resistance profiles of *Pseudomonas aeruginosa* strains isolated from dogs with otitis externa

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Abstract: *Pseudomonas aeruginosa* is an important pathogen that is frequently isolated from otitis cases in dogs, known to have high intrinsic and acquired resistance mechanisms to resist most antibiotics, and the ability to develop multiple antibiotic resistance. The aim of this study was to determine the antibiotic resistance profile of *P. aeruginosa* strains isolated from dogs with otitis externa symptoms. Totally 43 (26.70%) *P. aeruginosa* strains were isolated from 170 ear swab samples and all these strains were subjected to disk diffusion susceptibility testing and the resistance profiles of the strains were found to be as follows; 100% to trimethoprim sulfamethoxazole, 93.02% to doxycycline, 79.06% to neomycin, 58.13% to oxytetracycline, 34.88% to enrofloxacin, 25.58% to tobramycin, 20.93% to gentamicin, 13.95% to ciprofloxacin, 9.30% to amikacin, 4.65% to polymyxin B. Furthermore, multiple antibiotic resistance was detected in a total of 17 (39.53%) *P. aeruginosa* strains. As a result, it was understood that the most common Gram-negative bacteria isolated from otitis externa cases of dogs was *P. aeruginosa* and these isolates have shown high resistance to antibiotics from many antibiotic groups.

Keywords: Antibiotic resistance, Dog, Otitis externa, *Pseudomonas aeruginosa*.

Otitis eksternalı köpeklerden izole edilen *Pseudomonas aeruginosa* suşlarının antibiyotik direnç profilleri

Özet: *Pseudomonas aeruginosa*, köpeklerin otitis vakalarından sıklıkla izole edilen bir patojendir ve çoğu antibiyotiğe karşı birçok içsel ve edinilmiş direnç mekanizmasına sahip, çoklu antibiyotik direnci geliştirme kabiliyeti olduğu bilinen bir bakteridir. Bu çalışmanın amacı, otitis eksterna semptomları gösteren köpeklerden izole edilen *P. aeruginosa* suşlarının antibiyotik direnç profillerinin belirlenmesidir. 170 kulak sürüntüsü örneğinden toplam 43 (%26,70) *P. aeruginosa* suşu izole edildi ve bu suşların tümü disk difüzyon duyarlılık testine tabi tutuldu. İzole edilen *P. aeruginosa* suşlarının direnç profilleri trimetoprim sülfametoksazole %100, doksisikline %93,02, neomisine %79,06, oksitetrasikline %58,13, enrofloksasine %34,88, tobramisine %25,58, gentamisine %20,93, siprofloksasine %13,95, amikasine %4,30, polimiksin B'ye %4,65 şeklinde bulundu. Ayrıca toplam 17 (%39,53) *P. aeruginosa* suşunda çoklu antibiyotik direnci saptandı. Sonuç olarak köpeklerin otitis eksterna vakalarından en sık izole edilen Gram-negatif bakterinin *P. aeruginosa* olduğu ve bu izolatların farklı gruplardan antibiyotiklere yüksek direnç gösterdiği anlaşıldı.

Anahtar kelimeler: Antibiyotik direnci, Köpek, Otitis eksterna, *Pseudomonas aeruginosa*.

Introduction

Otitis externa is an acute or chronic inflammation of the external ear canal. The incidence of otitis externa in small animal medicine is around 20% (Cole 2004). Infectious otitis externa which is a multifactorial disease; arise as secondary complications of primary or predisposing causes such as atopic dermatitis, chronic irritation, conformational disorders, excessive moisture, obstructive causes, foreign bodies, metabolic diseases, ectoparasites, keratinization disorders (Graham-Mize and Rosser 2004).

The infectious agents that cause otitis externa are mostly bacteria, yeast and fungi (Shaw 2016). *Staphylococcus* spp, *Pseudomonas aeruginosa*, *Streptococcus* spp., *Proteus* spp., *Escherichia* coli, *Enterococcus* spp. are the most common bacterial agents that are frequently isolated from canine bacterial otitis externa cases (Petrov et al. 2013; Lee et al. 2019). Among yeasts such as *Malassezia pachydermatis* and *Candida* spp. are also frequently isolated from canine otitis externa cases with or without bacterial agents (Blanco et al. 2000; Yapicier et al. 2018).

P. aeruginosa is the most frequently isolated Gram-negative bacteria in canine otitis externa cases. *P. aeruginosa* is a rod shaped, aerobic, opportunistic bacteria which is frequently found in many environments such as soil, water, organic material and

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is not found in the normal ear flora of dogs (Morris et al. 2017; Pye 2018).

P. aeruginosa is intrinsically resistant to many antibiotic groups. This intrinsic resistance can be associated with different mechanisms such as low outer membrane permeability, chromosomal AmpC β-lactamase production, and the presence of a large number of genes encoding efflux pumps that cause multiple antibiotic resistance (Hancock 1998). *P. aeruginosa* may have resistance to most of penicillin, β-lactam group antibiotics, often aminoglycoside antibiotics, chloramphenicol and tetracyclines (Mekić et al. 2011; Penna et al. 2011).

Antibiotic resistance is a remarkable problem in both public and animal health. Increasing incidence of *P. aeruginosa* with increased intrinsic resistant strains and multiple antibiotic resistance makes it difficult to select antibiotics for treatment (Rubin et al. 2008; Pye 2018). Groups of antibiotics routinely used against *P. aeruginosa* infections in small animal medicine include aminoglycosides, fluoroquinolones, polymyxins, tetracyclines and potentiated sulfonamides (Martino De et al. 2016; Pye 2018). Making the selection of antibiotics used in the treatment according to the results of antibiotic susceptibility testing provides the increasing success of treatment regimes.

In this study, it was aimed to determine the prevalence of *P. aeruginosa* in otitis externa cases and the resistance of the strains against routinely used antibiotics.

Material and Method

Sample Collection

In this study, swab samples taken from the outer ear canal of 170 dogs with a clinical diagnosis of otitis externa sent from 13 veterinary clinics in the Ankara region between September 2019 and December 2020 were used. The selection of the dogs participating in the study was made by physical aurial examination (head shaking, aurial hyperemia and pruritus, discharge from the ear canal, unpleased odor, erythema, trauma due to scratching itself, pain on palpation, alopecia, ulceration, etc.) and otoscopic examination findings. Specimens were collected from the external ear canal of dogs using Amies Agar Gel Swabs (Oxoid, Hampshire, UK). The samples taken into the transport medium were kept at 4°C until meintened and processed within 24 hours. The swab samples have been examined in terms of both bacteriological and mycological aspects. *P. aeruginosa* AUVFM-07/01 strain obtained from Ankara University Faculty of Veterinary Medicine Department of Microbiology Culture Collection was used as positive control in all tests.

Bacteriological and Mycological Examination

In bacteriological examination, specimens which obtained using sterile swabs were inoculated on blood agar containing 5% sheep blood, MacConkey (MC) Agar (Oxoid CM0115) and Eosin Methylene Blue (EMB) agar (Oxoid CM0069B) and incubated at 37°C for 24-48 hours under aerobic conditions. Colonies were Gram stained and inspected according to their macroscopic and microscopic morphology. Alongside, biochemical tests such as catalase, oxidase, lactose fermentation, coagulase, indole, methyl red, Voges-Proskauer, citrate, urease, oxidation/fermentation tests were performed to identify genus and species. Cystine-Lactose-Electrolyte Deficient (CLED) Agar (Oxoid CM0301) was also used as a differential agar for the identification of pyocyanin formation of *P. aeruginosa*. Isolates were identified at the genus and species level using staining, biochemical tests and differential mediums (Holt et al. 1994; Quinn et al. 1994).

For mycological examination, collected swabs were inoculated on Sabouraud Dextrose Agar (SDA) (Oxoid CM0041) and incubated at 25°C for 2-4 weeks in an aerobic environment and colonies were controlled daily. Inoculations were also made on Chromogenic Candida agar (Oxoid CM1002) to differentiate *Candida* species in yeast isolations and incubated at 37-42°C for 48 hours. Mycological diagnosis was made by examining the incubation time and macroscopic/microscopic morphology of the colonies. In macroscopic examination, colonies were examined by considering their pigmentation and structure. In microscopic examination, colonies were stained with lactophenol cotton blue solution using cellophane tape method (Ghannoum and Isham 2009). Microscopically, hypha, septum, macroconidium, microconidium and spore structures belonging to fungal colonies were examined and diagnosed by classical mycological culture methods (Holt et al. 1994; Quinn et al. 1994).

Antibiotic Susceptibility Testing

Antibiotic susceptibility test was performed by the disk diffusion method recommended by Clinical & Laboratory Standards Institute (CLSI 2019). *P. aeruginosa* strains identified by conventional methods were inoculated on Mueller-Hinton (MH) agar (Oxo-

id CM0337). Antimicrobial susceptibility test disks (Oxoid, Hampshire, UK); gentamicin (CN10 µg), oxytetracycline (OT 30 µg), tobramycin (TOB 10 µg), polymyxin B (PB 300 U), enrofloxacin (ENR 5 µg), neomycin (N 30 µg), trimethoprim sulfamethoxazole (SXT 25 µg, 1.25 µg-23.75 µg), doxycycline (DO 5 µg), ciprofloxacin (CIP 5 µg), amikacin (AK 30 µg) were used. Agar plates were incubated at 37°C for 18-24 hours and inhibition zone diameters were measured for each disk according to CLSI standards (CLSI 2019; CLSI 2020). *Escherichia coli* ATCC 25922 strain was used as positive control in all tests. *P. aeruginosa* strains were defined as multi-drug resistant (MDR) in the case of resistance to one or more antibiotics from at least three different antibiotic groups (Magiorakos et al. 2012).

Results

Bacterial and Mycological Examination Results

No growth was observed in 9 (5.2%) of the 170 swab samples. *Staphylococcus* spp. (40.37%), *P. aeruginosa* (26.70%), *Streptococcus* spp. (18.01%), *E. coli* (4.96%), *Proteus* spp. (4.34%), *Corynebacterium* spp. (3.10%) and *Enterococcus* spp. (1.86%) were isolated among 161 samples.

Fungi and yeast growth were observed in 58 (34.11%) of 170 ear swab samples. According to the results 38 (65.51%) *Malassezia* spp., 11 (18.96%) *Candida albicans*, 5 (8.62%) *Aspergillus* spp. and 4 (6.89%) *Penicillium* spp. were isolated.

Antimicrobial Susceptibility Test Results

Among 43 *P. aeruginosa* strains; 43 (100%) of them were found resistant to trimethoprim sulfamethoxazole, 40 (93.02%) to doxycycline, 34 (79.06%) to neomycin, 25 (58.13%) to oxytetracycline, 15 (34.88%) to enrofloxacin, 11 (25.58%) to tobramycin, 9 (20.93%) to gentamicin, 6 (13.95%) to ciprofloxacin, 4 (9.3%) to amikacin and 2 (4.65%) to polymyxin B (Table 1). Some of these resistant strains of *P. aeruginosa* were found resistant to antibiotics from two or more antibiotic groups. Multiple antibiotic resistance profiles have been shown in Table 2.

Table 1. Antimicrobial resistance profiles of *P. aeruginosa* strains.

Antimicrobial Classes	Antimicrobial Agents	Number of Isolates (n =43)	Zone diameter breakpoint for resistant strains (mm)	Resistance (%)
Fluoroquinolones	CIP[b]	6	≤15	13.95
	ENR[a]	15	≤16	34.88
Aminoglycosides	AK[a]	4	≤14	9.3
	CN[a]	9	≤12	20.93
	TOB[b]	11	≤12	25.58
	N[a]	34	≤17	79.06
Polymyxins	PB[b]	2	≤11	4.65
Tetracyclines	DO[b]	40	≤18	93.02
	OT[b]	25	≤18	58.13
Sulfonamides	SXT[b]	43	≤23	100

n: Total number of isolates tested. AK: Amikacin, ENR: Enrofloxacin, N: Neomycin, OT: Oxytetracycline, TOB: Tobramycin, CN: Gentamicin, DO: Doxycycline, SXT: Trimethoprim-sulfamethoxazole, CIP: Ciprofloxacin, PB: Polymyxin B.

[a] Zone diameter breakpoints according to Performance Standards for Antimicrobial Disk and Dilution Susceptibility Test for Bacteria Isolated from Animals; Approved Standard Document 5th ed. CLSI supplement VET01S (CLSI, 2020).

[b] Zone diameter breakpoints according to Performance Standards for Antimicrobial Susceptibility Testing. 29th ed. CLSI supplement M100. Wayne, PA ABD (CLSI, 2019).

Table 2. Multiple antibiotic resistance profiles of *P. aeruginosa* strains.

Multidrug Resistance Profiles	No. of MDR Isolates	Prevalence of MDR Isolates
DO, SXT, N, ENR, OT	2	4.6
DO, SXT, N, ENR, AK	1	2.3
DO, SXT, N, PB, CN	1	2.3
DO, SXT, N, ENR	3	6.9
DO, SXT, N, OT	5	11.6
DO, SXT, N, AK	1	2.3
DO, SXT, OT, TOB	2	4.6
SXT, TOB, CIP, CN	2	4.6
Total:	**17**	**39.5**

Antimicrobial agent groups: Tetracyclines: DO, OT; Fluoroquinolones: ENR, CIP; Aminoglycosides: AK, CN, N, TOB; Sulfonamides: SXT; Polymyxins: PB.

Discussion and Conclusion

Otitis externa cases in dogs can occur due to primary and secondary causes (Shaw 2016; Pye 2018). It was thought that the 9 swab samples with no growth were considered to be according to primary non-infectious causes such as allergic conditions, autoimmune disorders, hormonal disorders, and obstructive causes. The most common bacterial agents isolated from otitis externa cases of dogs belong to *Staphylococcus* spp. (Blanco et al. 2000; Turkyilmaz 2008; Penna et al. 2011; Petrov et al. 2013; Bajwa 2019). In this study, the prevalence of *Staphylococcus* spp. from dogs with otitis externa was determined as 40.37%. This result supports the idea that *Staphylococcus* spp. is the most common bacteria isolated from dogs with otitis externa, as reported in other studies (Petrov et al. 2013; Paterson 2016; Korbelik et al. 2019). *P. aeruginosa* is not found in the normal ear flora of dogs, but it is frequently isolated from canine otitis cases. In this study, prevalence of *P. aeruginosa* (26.7%) was found to be high which is similar to Hariharan et al. (17.5%) (2006), Turkyilmaz (17%) (2008), Bugden (35.5%) (2013), Petrov et al. (17%) (2013), Bourély et al. (27.5%) (2019) and Dos Santos et al. (27.6%) (2019) findings.

P. aeruginosa is frequently isolated from otitis cases and treatment is often unsuccessful due to its high antimicrobial resistance (Ghannoum and Isham 2009; Mekić et al. 2011; Bourély et al. 2019; Chan et al. 2019). This is explained by the knowledge that *P. aeruginosa* is a bacterium that has an intrinsic antibiotic resistance mechanism and can exhibit MDR and can also easily develop resistance to new antibiotics (Hancock 1998). It is known that *P. aeruginosa* has high intrinsic resistance to tetracycline, sulphonamide and quinolone group antibiotics (Mekić et al. 2011; Penna et al. 2011; Arais et al. 2016). In this study, all *P. aeruginosa* strains were found to be resistant to trimethoprim-sulfamethoxazole from the sulfonamide group among the antibiotics tested from five different groups. In similar studies, Schick et al. (69%) (2007), Rubin et al. (57%) (2008), Ozturk et al. (100%) (2016) and Turkyilmaz (100%) (2008) reported that *P. aeruginosa* was highly resistant to trimethoprim-sulfamethoxazole.

In this study, *P. aeruginosa* strains resistant to oxytetracycline (58.1%) from tetracycline group is very similar to the findings of Ozturk et al. (50%) (2016) and the resistance to doxycycline (93%) is very similar to the findings of Martino et al. (78.3%) (2016) and Petrov et al. (78.3%) (2013).

The resistance of *P. aeruginosa* to antibiotics from the aminoglycoside group is provided by the inactivation of the antimicrobial agents by bacterial modifying enzymes (34). In this study, 90.7% *P. aeruginosa* strains were found to be susceptible to amikacin. Petrov et al. (100%) (2013), Schick et al. (81%) (2007) and Park et al. (97.8%) (2020) also found high sensitivity to amikacin in their studies. In this study, *P. aeruginosa* exhibited the highest sensitivity to amikacin among the aminoglycoside antibiotics tested. This is attributed to the opinion that amikacin has a low sensitivity to modifying enzymes, which is also reported in the literature (Poole 2005; Ekincioglu and Percin 2013).

Enrofloxacin is more lipophilic than ciprofloxacin (Davis et al. 2007). Therefore, efflux pumps, which are important in the elimination of fluoroquinolones, are known to more easily eliminate enrofloxacin (Wildermuth et al. 2007). Rubin et al. (2008) reported that *P. aeruginosa* strains isolated from dogs with diseases such as otitis, pyoderma, and soft tissue infections had 31% resistance to enrofloxacin and 16% resistance to ciprofloxacin. In this study, prevalence of resistance to enrofloxacin and ciprofloxacin was found to be 34.8% and 13%, respectively. Similar to this study, Park et al. (2020) reported that *P. aeruginosa* was more resistant to enrofloxacin than ciprofloxacin, thus supporting the view that *P. aeruginosa* exhibited higher resistance to enrofloxacin than ciprofloxacin.

P. aeruginosa strains isolated in this study exhibited high sensitivity (95.4%) to polymyxin B that has also been reported similarly in other studies (Tam et al. 2005; Bugden et al. 2013; Pye et al. 2013; Hyun et al. 2018). Polymyxin B is one of the limited

antibiotic options that can be used in the treatment of infections caused by Gram negative bacteria and especially *P. aeruginosa* that exhibit MDR, and is often preferred against *P. aeruginosa* in cases of otitis externa (Tam et al. 2005; Petrov et al. 2013). *P. aeruginosa* exhibits a very low intrinsic resistance to polymyxin B compared to other antibiotics and it is known that its ability to develop mutational resistance is limited (Fernández et al. 2013).

In this study, MDR prevalence (39.53%) of *P. aeruginosa* strains was found to be similar to the MDR prevalence of the studies reported by Dos Santos et al. (61.9%) (2019) and Eliasi et al. (92%) (2020). In these studies, sulphonamide, tetracycline, fluoroquinolone and aminoglycoside group antibiotics, which are known to exhibit intrinsic resistance in *P. aeruginosa*, were found at high levels in MDR profile combinations (Turkyilmaz 2008; Eliasi et al. 2020). In addition, the ability of *P. aeruginosa* to develop acquired resistance also contributes to the formation of MDR (Pang et al. 2019).

As a result, in this study which was conducted in the Ankara region, it was confirmed that the most common Gram-negative bacteria causing otitis externa in dogs was *P. aeruginosa*. In this study, it was observed that the resistance profile of *P. aeruginosa* strains isolated from otitis externa cases of dogs against antibiotics selected from antibiotic groups known to have resistance was consistent with similar studies. For a successful treatment of otitis externa in this region, it was thought that antibiotics such as amikacin and polymyxin B, which *P. aeruginosa* strains showed low resistance, could be preferred instead of trimethoprim-sulfamethoxazole, doxycycline and oxytetracycline, which *P. aeruginosa* strains show high resistance. The high prevalence of multiple antibiotic resistant strains emphasizes once again the importance of antibiotic susceptibility testing in the selection of antibiotics for treatment.

Conflict of Interest: The authors declare that they have no conflict of interest.

Ethical Statement: The materials used in this study are swab samples collected during clinical examination.

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