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Drug Resistance Pattern of *Pseudomonas aeruginosa* Isolates Carrying *MexAB-OprM* Efflux Pump's Associated Genes in Companion Birds with Respiratory Infection

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Abstract

BACKGROUND: *Pseudomonas aeruginosa* is considered one of the most common bacterial pathogens causing nosocomial infections in human cases. However, the pathogenesis of this bacterium in companion birds is poorly understood.

OBJECTIVES: The aim of the present study was to isolate *P. aeruginosa* from pet birds with respiratory illness manifestations referred to the clinic of the University of Tehran. Moreover, the antimicrobial susceptibility of the recovered *P. aeruginosa* isolates carrying MexAB-OprM efflux pump was evaluated.

METHODS: Selective media and biochemical tests were used to isolate and identify *P. aeruginosa* isolates from 126 companion birds. The species-specific polymerase chain reaction (PCR) based on the *16S rRNA* gene was applied to confirm *P. aeruginosa*. In addition, the sensitivity of isolates to 20 antimicrobial agents was assessed by an antimicrobial susceptibility test. Multiplex PCR was used to detect genes associated with MexAB-OprM efflux pump by specific primers in recovered *P. aeruginosa* isolates.

RESULTS: All seven isolates identified as *P. aeruginosa* in culture by biochemical tests were confirmed utilizing species-specific PCR. The results of the antimicrobial susceptibility test demonstrated multidrug resistance (MDR) among the isolates with the highest resistance to neomycin, kanamycin, rifampicin, and vancomycin (100% of isolates) followed by colistin (57% of isolates). The *mexA* and *oprM* genes were detected in all isolates by multiplex PCR, while the *mexB* gene was not amplified in any of the seven isolates.

CONCLUSIONS: We found *P. aeruginosa* isolates in sick birds and observed MDR in these isolates. Therefore, companion birds could be considered a potential public health concern, especially for owners and veterinary staff.

KEYWORDS: Antimicrobial susceptibility, Multiplex PCR, Nosocomial infections, Pathogenesis, Public health

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Introduction

Pseudomonas aeruginosa is a major pathogen in humans and animal species. This bacterium is a ubiquitous microorganism that remains alive under a wide range of environmental conditions. There are many reports of it causing different diseases in both livestock and companion animals (Kidd *et al.*, 2011; Poonsuk & Chuanchuen, 2012). Moreover, many studies noted the occurrence of *Pseudomonas* infection in various avian species mainly as an opportunistic pathogen (Abdul-Aziz, 2020). *Pseudomonas aeruginosa* is considered as the cause of some systemic diseases, the death of embryos and hatchlings, airsacculitis, sinusitis, keratitis, kerato-conjunctivitis, yolk sac infection, and septicemia in young birds (Abdul-Aziz, 2020; Razmyar & Zamani, 2016). The diagnosis of *Pseudomonas* is mostly based on isolation and molecular techniques. The low permeability of the cell membrane along with the efflux pumps has increased resistance to antibiotics in *P. aeruginosa*. As a result, antibacterial susceptibility tests are recommended before starting the treatment (Loughlin *et al*., 2002).

The most important mechanism involved in the antimicrobial resistance of *P. aeruginosa* is the reduced concentration of antimicrobials in the intracellular fluid. The expression of extracellular beta-lactamase from both plasmid and genomic DNA along with enzymes altering the chemical structure of aminoglycosides and ultimately, decreased cell membrane permeability improves the antimicrobial resistance of *P. aeruginosa* (Aeschlimann, 2003). The MexAB-OprM pump is composed of three parts: 1) a surface protein embedded in the cell membrane called multidrug resistance (MDR) protein MexA, 2) MDR protein MexB responsible for the active transport of antimicrobial agents, and 3) transmembrane protein channels known as outer membrane protein OprM involved in sending the substances out of the cell (Mokhonov *et al*., 2004).

Pseudomonas infection in pet birds leads to upper and lower respiratory tract infections, such as rhinolith, sinusitis, and tracheitis (Harcourt-Brown & Chitty, 2005). There is growing attention to this pathogen because of various clinical signs in companion birds and antimicrobial drug resistance among different strains. The occurrence of *P. aeruginosa* in pet birds in Iran is poorly investigated. Therefore, further studies are required to provide sufficient information on the prevention and treatment of this pathogen. We isolated *P. aeruginosa* from companion birds with upper respiratory tract infection in Iran as the first study on the efflux pump family genes in companion birds associated with *Pseudomonas* infection. In this study, the effectiveness of diverse antimicrobial agents was also evaluated against this microorganism using the agar disk diffusion method.

Materials and Methods

Sampling

A total of 126 companion birds from different orders, including passeriformes, psittaciformes, columbiformes, and galliformes, which referred with clinical signs to the pet bird clinic of the University of Tehran during February 2018-May 2018 were sampled. Birds with symptoms related to the respiratory system, such as sinusitis, nasal discharge, wheezing, or eye discharge were selected. Swab samples were taken from the eye or choanal slit of each bird and were incubated in enriched tryptose soy broth (TSB) at 37ºC for 24 h (Quinn *et al*., 2002).

Aliquots of TSB were plated onto both MacConkey agar and blood agar, followed by incubation at 37ºC for 24 h. Colonies were observed by optical microscope after Gram staining to detect *Pseudomonas* microorganisms. Biochemical tests, including gas production, triple sugar iron (TSI), and motility were performed. The confirmed *P. aeruginosa* isolates were kept frozen at -70ºC until future use. All media were purchased from HiMedia Laboratories (Pvt. Ltd, India).

Drug Resistance

The resistance of *P. aeruginosa* isolates to the number of antibacterial medications (Table 1) was determined by the agar disk diffusion method. The results were interpreted based on the Clinical and Laboratory Standards Institute guidelines (CLSI, 2008). The used antibacterial drugs and respective concentrations were amikacin, neomycin, nalidixic

acid, tetracycline, gentamycin, ciprofloxacin, vancomycin, norfloxacin, streptomycin, enrofloxacin, levofl-oxacin, kanamycin, danofloxacin, lincospectin, trimethoprim+ sulfamethoxazole, colistin, ofloxacin, meropenem, rifampicin, and cefotaxime at the concentrations of 30, 30, 30, 30, 10, 5, 30, 10, 10, 5, 5, 30, 10, 15/200, 1.25/23.75, 10, 5, 10, 5, and 30 µg, respectively.

All antibacterial disks were provided by Padtan Teb Co. (Tehran, Iran). The ATCC reference strains *Escherichia coli* ATCC 25922, *P. aeruginosa* ATCC 27853, and *E. coli* ATCC 35218 were utilized for quality control purposes. In this study, the *P. aeruginosa* isolates with intermediate susceptibility classification were considered not resistant to that medicine, and multi-resistance was defined as resistance to more than one agent.

Molecular Detection

A single polymerase chain reaction (PCR) was employed to confirm the detection of *P. aeruginosa*. Furthermore, multiplex PCR was used to detect the presence of genes encoding MexAB-Oprm efflux pump involved in antimicrobial resistance. For all PCRs, bacterial DNA was extracted from each isolate using the boiling method as follow: two or three fresh colonies of *P. aeruginosa* were suspended in 500 µL sterile distilled water and then boiled for 10 min at 100ºC. Afterwards, the mixture was centrifuged for 5 min and the supernatant containing chromosomal DNA was collected for PCR.

Bacterial isolates identified as *P. aeruginosa* by both bacteriological and biochemical tests were subjected to species-specific (SS) PCR using *16S rRNA* set of primers as forward (5'- GGGGGATCTTCGGACCTCA-3') and reverse (5'-TCCTTAGAGTGCCCACCCG-3') (Spilker *et al.,* 2004). Amplification reactions were carried out in a 50 µL reaction volume containing 25 µL of 2x master mix (Ampliqon, Denmark), 1 µL (100 pmol) of each of forward and reverse primers (with 10 pmol concentration), and 19 µL deionized H_2O. Approximately 100 ng of template DNA (4 µL) was added to the mixture. Negative controls (dH_2O instead of template DNA) were included in all PCR reaction sets. The amplification program in the thermocycler (SensoQuest, Germany) was 95°C for 2 min followed by 25 cycles of 94°C for 20 s, 59°C for 20 s, 72°C for 40 s, and a final extension at 72°C for 1 min. Agarose gel electrophoresis was used to show the amplified DNA bands in 1% agarose gel at 70 V for 80 min in 1x TAE buffer.

Genes encoding MexAB-OprM efflux pump were detected by specific primers targeting *mexA*, *mexB*, and *oprM* antimicrobial resistance (AR) genes using multiplex PCR. The sequences of primers applied to amplify *mexA*, *mexB*, and *oprM* genes are shown in Table 2 (Arabestani *et al.*, 2015). Amplification reactions were carried out in a 50 µL reaction volume containing 25 µL of 2x master mix (Ampliqon), 2 µL of each primer set, and 19 µL dH_2O. Approximately 100 ng of template DNA (4 µL) was added to the mixture. In all PCR reaction sets, the standard strain of *P. aeruginosa* as positive control and dH_2O instead of template DNA as negative controls were included. The amplification program in the thermocycler (SensoQuest, Germany) was as follow: 94°C for 5 min followed by 30 cycles of 94°C for 30 s, 67°C for 30 s, 72°C for 1 min, and a final extension at 72°C for 5 min. Agarose gel electrophoresis was used to show the amplified DNA bands in 1% agarose gel at 70 V for 90 min in 1x TAE buffer.

Results

Bacteriological Findings

A total of seven *P. aeruginosa* isolates were identified from swab samples using both bacteriological and biochemical tests. These isolates were from different bird species, including five domestic canaries, one pigeon, and one cockatiel.

Drug Resistance

The antimicrobial susceptibility test indicated that *P. aeruginosa* isolates from companion birds were 100% resistant to neomycin, kanamycin, rifampicin, and vancomycin (Table 1). No resistance was observed to 13 out of 20 antimicrobial agents. Four resistance patterns were found for seven isolates, three isolates of which (42.8%) had pattern 1 and two isolates (28.6%) had pattern 2 (Table 3). Each of the two remaining isolates belonged to a single pattern. All isolates were the MDR type with variable resistance to 4-6 antimicrobial agents (Table 4).

Table 1. Antimicrobial susceptibility of seven *Pseudomonas aeruginosa* isolates

No.	Antimicrobial agent	Resistant	Susceptible
		No. (%)	No. (%)
1	Streptomycin	3 (42.8)	4 (57.2)
2	Levofloxacin	7 (100)	0 (0)
3	Danofloxacin	7 (100)	0 (0)
4	Norfloxacin	7 (100)	0 (0)
5	Cefotaxime	1 (14.3)	6 (85.7)
6	Kanamycin	0 (0)	7 (100)
7	Linco-spectin	7 (100)	0 (0)
8	Gentamycin	7 (100)	0 (0)
9	Ciprofloxacin	7 (100)	0 (0)
10	Vancomycin	0 (0)	7 (100)
11	Amikacin	7 (100)	0 (0)
12	Tetracycline	7 (100)	0 (0)
13	Neomycin	0 (0)	7 (100)
14	Nalidixic acid	0 (0)	7 (100)
15	Trimethoprim-Sulfamethoxazole	7 (100)	0 (0)
16	Ofloxacin	7 (100)	0 (0)
17	Meropenem	7 (100)	0 (0)
18	Colistin	3 (42.8)	4 (57.2)
19	Enrofloxacin	7 (100)	0 (0)
20	Rifampicin	0 (0)	7 (100)

Table 2. The sequences of primers used in this study

Gene	Sequence (5'–3')	Product size (bp)
MexA-F	CTCGACCCGATCTACGTC	503
MexA-R	GTCTTCACCTCGACACCC	
MexB-F	TGTCGAAGTTTTTCATTGAG	280
MexB-R	AAGGTCACGGTGATGGT	
OPrM-F	GATCCCCGACTACCAGCGCCCCG	247
OPrM-R	ATGCGGTACTGCGCCCGGAAGGC	

Table 3. Drug resistance patterns among seven *Pseudomonas aeruginosa* isolates

Pattern #	Resistant to	No. of isolates (%)
1	Kanamycin, Vancomycin, Neomycin, Rifampicin	3 (42.8)
2	Kanamycin, Vancomycin, Neomycin, Rifampicin, Colistin	2 (28.6)
3	Kanamycin, Vancomycin, Neomycin, Rifampicin, Colistin, Nalidixic acid	1 (14.3)
4	Kanamycin, Vancomycin, Neomycin, Rifampicin, Colistin, Streptomycin	1 (14.3)

Table 4. Multidrug resistance among seven *Pseudomonas aeruginosa* isolates

No. of resistant isolates (%)	No. of antimicrobial agents
7 (100%)	4 ≤
4 (57%)	5 <
2 (28%)	6 <

Molecular Detection

By PCR and specific primers for 16S rRNA, a 965 bp fragment was amplified in all the seven isolates and positive control followed by detection using agar gel electrophoresis. This observation confirmed the presence of *P. aeruginosa* (Figure 1). Multiplex PCR was employed in order to detect the resistance genes encoding the mexAB-OprM efflux pump. For each isolate, the reaction was repeated at least four times. The results showed 503 bp and 247 bp PCR products in all these isolates indicating the presence of *mexA* and *oprM*, respectively. Neither in clinical nor in positive control isolates, any fragment representing the *mexB* gene was detected (Figure 2).

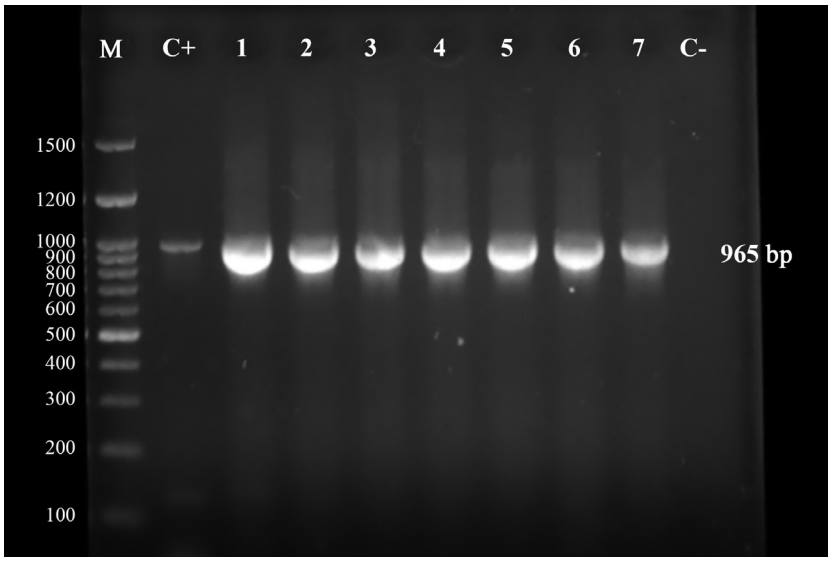

Figure 1. Electrophoresis of PCR-amplified 16 rRNA gene of *Pseudomonas aeruginosa* field isolates on %1 agarose gel. Amplified 965 bp bands of field isolates are shown in lanes 1 to 7. Lanes M, C+, and C- indicate 1 kb ladder, positive control, and negative control (dH2O instead of cDNA), respectively.

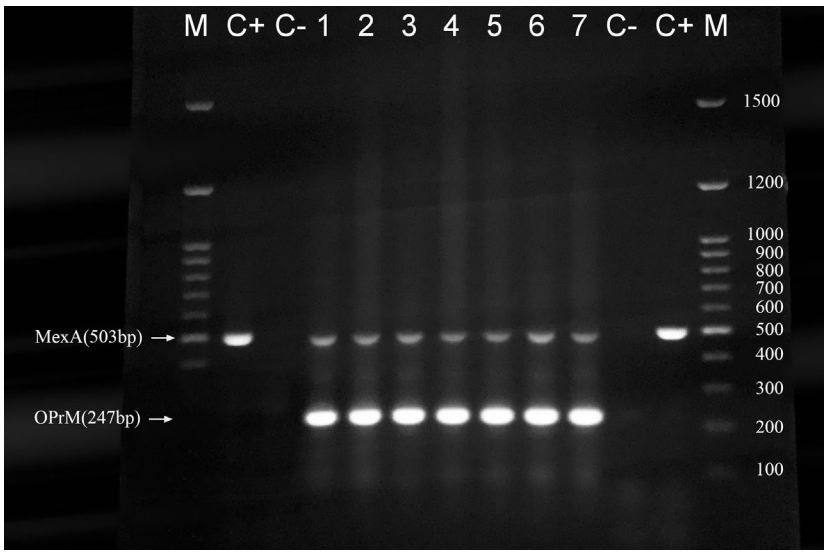

Figure 2. Electrophoresis of PCR-amplified mexAB and OprM genes of *Pseudomonas aeruginosa* field isolates on %1 agarose gel. Amplified 503 bs (for MexA gene) and 247 (for OPrM gene) bp bands of field isolates are shown in lanes 1 to 7. Lanes M, C+, and C- indicate 1 kb ladder, positive control, and negative control (dH2O instead of cDNA), respectively.

Discussion

Pseudomonas aeruginosa can be found everywhere allowing the organism to spread through various routes. The pathogenicity of *P. aeruginosa* is mostly associated with opportunistic infection. This finding emphasizes the role of other microorganisms in enhancing susceptibility to *P. aeruginosa*. Infection due to *P. aeruginosa* in companion birds is a great danger not only for the life of the birds but also for the health of its owner. Reports on *Pseudomonas* infection in companion birds have been reviewed recently (Abdul-Aziz, 2020).

Pseudomonas aeruginosa can be freely transferred from birds to humans and vice versa. The severity of infection and disease outcome depend on the health status of the bird, pathogen virulence, and delay in treatment. The MDR capability of *P. aeruginosa* made the treatment of this ubiquitous organism even harder. The presence of the MexAB-OprM efflux pump complex encoded by related genes plays a key role in the determination of the level of antimicrobial resistance of *P. aeruginosa* (Terzi *et al*., 2014).

The findings of the present study revealed the presence of both MexA and OprM in all isolates, while MexB was not detected in any of these isolates. The high expression of mexR and OprD was reported in different *P. aeruginosa* isolates recovered from hospital patients using qPCR (Arabestani *et al*., 2015). Some studies discovered the lack of efflux genes among some *P. aeruginosa* clinical isolates and the majority of the rest (60%) demonstrated the combination of *mex-B* and *oprD* genes (Zaki *et al*., 2017). The overexpression of the MexAB-Oprm efflux pump has been reported to elevate the resistance of *P. aeruginosa* isolates to antimicrobial agents, such as carbapenem, amikacin, gentamicin, ciprofloxacin, and meropenem with a range of 32%-84.5% (Pan *et al*., 2016; Pourakbari *et al.*, 2016).

Although the important role of the MexAB-Oprm efflux pump in the resistance of *P. aeruginosa* to antimicrobial agents has been shown, the function of other mechanisms in the development of resistance should not be ignored. To illustrate, the mutations in distinct regions of *MexR* and *NaID* have been found to upregulate the *mexA* gene, and also the high expression of OprD can result in resistance to carbapenems (Arabestani *et al.*, 2015; Pan *et al.*, 2016).

In the present study, all seven isolates were resistant to neomycin, kanamycin, rifampicin, and vancomycin. However, no resistance to meropenem and fluoroquinolones, namely ciprofloxacin, danofloxacin, norfloxacin, ofloxacin, and enrofloxacin was observed. The high susceptibility to fluoroquinolones was also reported previously in *P. aeruginosa* isolates from companion birds (Yakimova *et al.*, 2016). Contradictory findings on antimicrobial susceptibility were represented in studies conducted on *P. aeruginosa* isolated from nosocomial infections in which high resistance was reported against meropenem, ciprofloxacin, amikacin, and gentamicin (Doosti *et al.*, 2013; Arabestani *et al*., 2015; Hashemi *et al*., 2016: Pourakbari *et al*., 2016; Zaki *et al*., 2017; Ghasemian *et al*., 2018).

The difference in antimicrobial susceptibility might be due to the absence of the *mexB* gene in isolates of our study. In addition, high susceptibility to fluoroquinolones was reported in benzalkonium chloride-adapted *P. aeruginosa* cells (Loughlin *et al.*, 2002). These results imply that *P. aeruginosa* isolated from nosocomial infections are more resistant to antimicrobial agents. It might be due to the broad usage of disinfectants causing alteration in cell surface structure and the overexpression of MexAB-OprM efflux pumps.

Conclusion

In conclusion, *P. aeruginosa* can be considered as one of the causative pathogens of upper respiratory problems in companion birds. Seven isolates of *P. aeruginosa* in this study showed distinct antimicrobial susceptibility patterns, compared to those of *P. aeruginosa* isolated from nosocomial infections in human cases. It indicates variable drug resistance profiles among *P. aeruginosa* isolates originated from different sources of infection. Therefore, an antibacterial susceptibility test prior to the administration of any antimicrobial agent to patients is important for avoiding an increase in resistance. Concurrent infections with other pathogens of the respiratory tract and transmission from humans or other animals along with environmental factors should be considered in *P. aeruginosa* infections.

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Conflict of Interest

The authors declared no conflict of interest.

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مجله طب دامی ایران، ۱۴۰۰، دوره ۱۵، شماره ۴، ۳۷۸-۳۸۶

الگوی مقاومت داروئی جدایه‌های سودوموناس اروژینوزا (*Pseudomonas aeruginosa*) حامل ژن‌های مرتبط با پمپ برون‌ریز MexAB-OprM به‌دست‌آمده از پرندگان زینتی درگیر عفونت تنفسی

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زمینه مطالعه: *سودوموناس اروژینوزا* (*Pseudomonas aeruginosa*) از معمول‌ترین پاتوژن‌های باکتریایی مسبب عفونت‌های بیمارستانی در انسان است. اما در خصوص بیماری‌زائی این پاتوژن در پرندگان زینتی اطلاعات کمی در دسترس است.

هدف: این مطالعه به‌منظور بررسی جداسازی *سودوموناس اروژینوزا* از پرندگان زینتی درگیر عفونت تنفسی ارجاع شده به کلینیک و سپس ارزیابی حساسیت ضدمیکروبی جدایه‌های *سودوموناس اروژینوزا* حامل ژن‌های پمپ برون‌ریز MexAB-OprM بود.

روش کار: محیط‌های انتخابی، آزمایشات بیوشیمیائی برای جداسازی و شناسائی *سودوموناس اروژینوزا* از ۱۲۶ پرنده زینتی مورد استفاده قرار گرفت. واکنش PCR اختصاصی گونه بر اساس ژن *16S rRNA* برای تائید گونه *سودوموناس اروژینوزا* به کار برده شد. علاوه بر آن، آزمایش حساسیت ضدمیکروبی برای ارزیابی حساسیت جدایه‌ها به ۲۰ عامل ضد میکروبی انجام شد. از روش Multiplex PCR و به کارگیری پرایمرهای اختصاصی، ژن‌های پمپ برون‌ریز مورد جستجو قرار گرفت.

نتایج: نتایج آزمایش حساسیت ضدمیکروبی مقاومت چندگانه بین جدایه‌ها را نشان داد با بیشترین میزان مقاومت به نئومایسین، کانامایسین، ریفامپیسین و ونکومایسین (۱۰۰٪) و بعد از آن به کلیستین (۷۵٪). ژن‌های *mexA* و *oprM* در تمامی جدایه‌های مورد مطالعه در این پژوهش مشاهده شد اما ژن *mexB* در هیچ‌کدام از هفت جدایه یافت نشد.

نتیجه‌گیری نهایی: بر اساس نتایج این مطالعه، آلودگی پرندگان زینتی به *سودوموناس اروژینوزا* و مشاهده مقاومت داروئی چندگانه در بین آنها از نقطه نظر بهداشت عمومی به‌ویژه برای صاحبان پرنده و همکاران دامپزشکی حائز اهمیت است.

واژه‌های کلیدی: بهداشت عمومی، بیماری‌زائی، حساسیت ضدمیکروبی، عفونت‌های بیمارستانی، مولتی‌پلکس PCR

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