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292

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RESEARCH ARTICLE

Otitis External Infections Among Jordanian Patients with Emphasis on Pathogenic Characteristics of *Pseudomonas aeruginosa* Isolates

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Abstract:

Introduction:

Otitis external infection is an inflammation of the ear canal frequently caused by *Pseudomonas aeruginosa*, followed by *Staphylococcus epidermis* and *Staphylococcus auerus.*

Objective:

This study investigated the spectrum of bacterial and fungal agents that cause otitis external infection in Jordanian patients with an emphasis on important antimicrobial resistance genes and putative virulence factors of *P. aeruginosa* isolates using molecular PCR methods.

Methods:

A total of 128 ear swab samples were obtained from outpatients with otitis external infection of Ear-Nose-Throat Clinic (ENT) from the Jordan University Hospital (JUH). All samples were cultured for bacteria and fungi and their growth was identified by macroscopic and microscopic examination as well as recommended biochemical tests.

Results:

Positive growth of bacteria and fungi were found in 105/128 (82%) of the examined cases. A total of 28 (22%) of the recovered organisms from ear samples were *P. aeruginosa*. A total of 11/28 (39%) of *P. aeruginosa* isolates were Multidrug-Resistant (MDR) which are resistant to three or more antibiotic classes. Both *blaIMP-15* and *VIM* genes were not detected, while *KPC genes* were found in 57% among all isolates. The rates of the potential virulence genes found among 28 *P. aeruginosa* isolates were as follows: *las*B, *alg*D, *tox*A, *exo*U *PilB* and *exo*S at 100%, 100%, 82%, 72%, 54% and 25%, respectively. All isolates produced beta hemolysis on both human and sheep blood agar and showed either the pigment pyoverdin (57.1%) or pyocyanin (42.8%).

Conclusion:

Accurate identification of the causative agent of otitis external infection and its susceptibility to antibiotics especially *P.aeruginosa* is highly important for successful treatment. No significant relationship has been found between MDR *P. aeruginosa* and the presence of virulence genes.

Keywords: Otitis externa, Jordanian patients, Causative organisms, *P. Aeruginosa*, Antimcicrobial resistanc, Virulence factors.

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1. INTRODUCTION

Otitis external infection is a common clinical feature observed as an acute or chronic disease [1]. Most studies reported that otitis externa is frequently caused by *Pseudomonas aeruginosa*, followed by *Staphylococcus epidermidis* and *Staphylococcus aureus*. Almost 50% percent of otitis external cases caused by a single organism and others caused by two organisms include a variety of *bacteria* spp. and they are less frequently caused due to fungal agents such as *Aspergillus* spp. and *Candida* spp [1 - 4].

Otitis external infection caused by *Staphylococcus* spp. can easily be treated with a single antibiotic, whereas, infection due to *P. aeruginosa* requires a combination of topical antibiotics, such as aminoglycosides, polymyxin B, or fluoroquinolones

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with steroid preparations. However, treatment with systemic antibiotics is rarely needed for acute otitis external infection [1 - 5].

More recent studies from Jordan and other Middle Eastern Arab countries have shown that *P. aeruginosa* isolates from clinical specimens were commonly Multidrug Resistant (MDR) to many frequently used antibiotics in clinical medicines such as amikacin, aztreonam, gentamicin, piperacillin-tazobactam and ciprofloxacin, while they were mostly susceptible to imipenem, ceftazidime and colistin B [6 - 9]. Many studies have recommended treating patients with colistin B, gentamicin or ciprofloxacin topical preparations as the first-line treatment of otitis externa [2, 5, 10].

The prevalence of Extended-Spectrum β-Lactamases (ESBLs), carbapenemases (KPC), and Metallo-Beta-Lactamases (MBLs), especially VIM, IMP among *P. aeruginosa* has increased in recent years over the world including the Middle Eastern countries [6, 7, 11, 12].

P. aeruginosa carried a variety of virulence factors which allow the organism to develop biofilm and adhere to infected tissue surfaces, increase its survival rate and induce damage in the infected host. The most important of these virulence factors are exopolysaccharide called alginate lyase enzyme, Las B elastase, exotoxin A, Exoenzyme S and U [6, 11, 12].

This study investigated the spectrum of microbial causative agents of otitis external infections among Jordanian patients, with emphasis on *P. aeruginosa* and its association with antimicrobial susceptibility pattern and virulence genes

2. METHODS

This prospective convenience sampling study was conducted on patients presented to ENT Clinics of The Jordan University Hospital (JUH), and who were diagnosed with otitis external infections over the period from January 2017 through September 2017. A total of 128 patients were included, and specific biographical data of each patient was recorded on special forms as part of routine clinical investigation. The form included age, gender, name, and duration of hospitalization, disease history, clinical diagnosis, and general treatment with antibiotics if they were consumed was at the time of sampling the specimens.

This study was first approved by the Schools of Medicine and Graduate Studies, The University of Jordan, Amman, Jordan. The ethical approval was obtained from the Institutional Review Board (IRB) and The ethical committee of the Jordan University Hospital (2/2017, 10/1/2017), and signed consent was obtained from each examined patient.

Two clinical samples were collected from the discharge and external auditory canals of each patient with symptoms of otitis external infection using one cotton swab carried in transport medium, and the second was a wet swab immersed in physiological saline. The first swab was cultured on blood, chocolate agar, MacConkey agar and Sabouraud dextrose agar (Oxoid, England). The second swab was used for wet preparation and Gram-stain. All positive bacterial cultures were first identified using Gram-stain, oxidase and catalase tests, secondly, they were identified according to conventional methods described by Baily & Scott's/Diagnostic Microbiology[13] and few isolates were identified using ViteK 2 System (Biomeriex, France). All culture plates were incubated for 2 days at 37°C.

Fungal positive cultures were identified by macroscopic and microscopic examination and suspected *Candida* isolates by using further CandidaChrom agar (Oxoid, England) and germ tube test [13]. All fungal culture plates were incubated additionally for 7 days at room temperature (20-24 °C). First identified *P.* aeruginosa isolates were subcultured on Cetrimide Pseudomonas Selective Agar Base (Merck, Germany), incubated for 24-48 hours at 37 °C, and examined for the presence of blood hemolysis, fluorescent-colored colonies with yellow or blue-green pigmentation. All *P. aeruginosa* isolates were inoculated and stored in cryogenic tubes containing brain-heart infusion broth with 20% glycerol at -75 ºC for further investigation and were confirmed as *P.aeruginosa* using specific primers and PCR.

2.1. Antimicrobial Susceptibility Using Disc Diffusion Method

Confirmed *P. aeruginosa* isolates were first examined for antimicrobial susceptibility using disc diffusion test according to the guidelines of the Clinical Laboratory and Standard Institute (CLSI, 2015) [14]. Second, all MDR *P. aeruginosa* isolates have their minimum inhibitory concentrations (MICs) tested by E-test for ceftazidime, colistin, amikacin, azetronam, imipenem. The antimicrobial susceptibility results were interpreted in accordance with CLSI [14]. *P.aeruginosa* ATCC 27853 was included as a control strain during all tests.

P. aeruginosa isolates stored in cryotubes at -70ºC were thawed at room temperature, and cultured on blood agar. After incubation at 37ºC for 24 hours, a few colonies were selected from the agar, inoculated into Mueller Hinton broth and incubated at 37ºC for 18 hours, using the Wizard genomic DNA Purification Kit, Promega (USA) according to the instructions of the manufacturer the bacterial DNA was extracted. The bacterial plasmid was extracted using the EZ-10 Spin Column Plasmid DNA Minipreps Bio Basic kit (Canada) according to the manufacturer's instructions. Two PCR assays were performed; one is specific for the genus *Pseudomonas*, while the other is specific for *P. aeruginosa*, and two pairs of primers were used for each assay based on 16S ribosomal DNA (rDNA) sequence as reported by Spilker *et al*. [15]. Virulence genes *(alg* D, *las* B, *tox* A) were detected as described by Wolska *et al*. [16], whereas, virulence genes (*exo* S *and exo* U) were detected as reported by Mitov *et al*. [17].

BlaKPC genes among *P. aeruginosa* isolates were detected as reported by Akpaka *et al.* [18], while the two MBLs genes (*blaIMP* and *blaVIM)* were detected as described by Pitout *et al*. [19].

2.2. Statistical Analysis

Data generated from the study were tabulated on Microsoft Excel sheet and uploaded to the Statistical Package for Social Sciences, version 20 (IBM Corp, Armonk, NY, USA). The frequencies and percentages were calculated for categorical data. Pearson's chi-squared test or Fisher's exact test were

applied to determine potential factors associated with *P. aeruginosa* and to determine whether there are any statistical differences between the groups. The level of significance was set at a *p*-value of 0.05 to test the hypothesis without association. Fisher's exact test replaces the chi-squared test when the minimum expected count is less than five patients.

3. RESULTS

The demographic characteristics and clinical features of 128 examined patients are presented in Table **1**. The spectrum of microorganisms isolates from ear samples was 105/128 (82%) as shown in Table **2**. The list included all bacterial isolates 68/82 (82.9%) which revealed significant growth based on the number of detected bacterial colonies (≥10) in culture plates. Fungal isolates accounted for 14/82 (17.1%), and mixed cultures 17/82 (20.7%) were defined as one significant bacterial type isolate (≥10), and the second with few colonies less than 10 of any organism.

A total of 28 (22%) of *P. aeruginosa* isolates were recovered and confirmed by biochemical tests and PCR. A yellow-green pigment (pyoverdin) was found in 16/28 (57%), while the production of the blue-green pigment (pyocyanin) was observed in 12/28 (43%). All *P. aeruginosa* isolates have shown complete hemolytic activity on both human and sheep blood agar plates after 48 hrs of incubation at 37ºC.

The antimicrobial susceptibility patterns of the 28 *P. aeruginosa* isolates are shown in Table **3**. Ceftazidime, ciprofloxacin, imipenem and azetreonam were highly to moderately susceptible in the range of (82-68%), respectively, while the antimicrobial drugs; gentamicin and amikacin indicated high rates of resistance in the range of (72-57%), respectively. Only one isolate was resistant to colistin. Minimum inhibitory concentration ranges (MIC_{50} and MIC_{90}) for 4 tested antibiotics are presented in Table **3**. The most common virulence genes detected among *P. aeruginosa* isolates were *algD* and *lasB* (100%), followed by *toxA* gene (82%), *exo*U (72%), *pilB* (54%), *exo*S(25%) (Table **4**). While 16/28(57%) of *P. aeruginosa* isolates were positive for potential *KPC* genes, but all were negative for the presence of potential *VIM-2 and IMP-15* genes (Table **4**).

4. DISCUSSION

The present study showed a wide spectrum of 19 bacteria and fungi species recovered from ear samples of patients with otitis externa. The two most common organisms were *P. aeruginosa* and *S.* coagulase-negative, each accounted for 22% of isolates, whereas, fungal isolates were presented only by 17%. The majority of the otitis externa cases are caused by single bacterial pathogen as shown in Table **2**. Additionally, it has been found that 17.9% of the samples were negative for any growth. The reason for such negative cultures is most probably due to the treatment of patients with antibiotics before taking their ear samples (Table **1**).

A five-year retrospective study from New Zealand (2007-2011), with recorded data of 347 patients with otitis externa from Wellington Hospital, showed that *P. aeruginosa* was the most common organism (46.5%), while *S. aureus* was the second most common (31.9%) [3]. Most studies published during the last 10-years from various countries reported that microbial otitis externa is frequently caused by *P. aeruginosa* and less frequently by other Gram-negative bacteria and *S. aureus* and fungi [3, 5, 9, 20].

Table 1. Demographic characteristics of 128 investigated patients.

Characteristics	Male No. (%)	Female No. (%)	Total No (%)	P-Value	Odds Ratio (95% CI)
Age (mean ±SD) years	43.4±42.6	37.7±16.9	40.6±19.2	0.149	–
Age range (years)	1.5- 80 y	8 m - 66 y	8 m -80 y	–	–
Presence of ear discharge	51 (64.6)	33 (67.3)	84 (65.6)	0.747	0.883 (0.415,1.878)
Treatment with Antibiotic	53 (67.1)	34 (69.4)	87 (67.9)	0.786	0.899 (0.417,1.937)
No antibiotics were taken	26 (32.9)	15 (30.6)	41 (32)	–	–
Total	79 (61.7)	49(38.3)	128(100)	–	–

Table 2. Types of organisms isolated from ear discharges of 128 patients.

Microorganism	No. (%) of Isolates
Bacteria	
*Pseudomonas aeruginosa**	28(22)
Pseudomonas putida	2(1.5)
Streptococcus Viridans	2(1.5
Streptococcus pyogenes	1(0.8)
Staphylococcus Coagulase- negative**	28(22)
Staphylococcus auerus	14(10.9)
Klebsiella pneumoniae	4(3.1)
Citrobacter koseri	1(0.8)
Sphingomonas paucimobilis	1(0.8)
Enterobacter cloacae	1(0.8)
Moraxella lacunata	1(0.8)
Rhizobium radiobacter	1(0.8)
Hemophillus influenza type b	1(0.8)
Corynebacteria spp.	5(3.9)
Enterococcus fecalis	1(0.8)
Fungi	
Candida albicans	8(6.3)
Asperigullus flavus	3(2.3)
Asperigillus niger	2(1.5)
Pencillum species	1(0.8)
Total growth	105(82.04)
No growth	23(17.96)
Mixed sample **	17(20.7)

* Pyoverdin was observed in 57.2%; pyocyanin) was observed in 42.8% of the isolates.

**Mostly two types of bacteria species.

Two old Jordanian studies have reported the spectrum of organisms isolated from a general ear infection as follows. The first study has investigated patients with otitis externa in the South of Jordan, and it has reported that positive culture results accounted for the following: *P. aeruginosa* (39%), *Aspergillus*

spp (27%), *Candida albicans* (18%), *S. aureus* (18%), and no growth was detected in 8.5% [21]. The second study has investigated all ear infections in the Jordanian city Al-Zarqa and indicated that *P. aeruginosa* was found in 41.7%, followed by *Aspergillus species* (19.4%), *Candida albicans* (10.6%), *S. aureus* (16.1%) and *Proteus mirabilis* (2.8%) [22]. Both studies have shown higher percentages of positive fungi isolates than this study.

Table 3. Antimicrobial susceptibility pattern and MICs of 28 *P. aeruginosa* isolates.

Antimicrobial Agents	No.(%) susceptible	MIC_{90} (µg/ml)	MIC Range (µg/ml)
Colistin	27(96)	0.32	0.32-2
Ceftazidime	23(82)	9.8	0.125– >256
Ciprofloxacin	22(79)	-	-
Imipenem	21(75)	2.6	0.25 – 24
Piperacillin-tazobactam	21(75)	-	-
Aztreonam	19(68)	5.3	0.25 – 16
Meropenem	19(68)	-	-
Amikacin	12(43)	13.6	1.5 – >256
Gentamicin	8(29)	-	

Table 4. Distribution of potential virulence genes, *blaKPC* and MBLs genes among 28 *P. aeruginosa* isolates.

Virulence Genes	No. (%) Positive Virulence Genes	No. (%) MDR Isolates*
Elastase B (*lasB)*	28(100)	11(100)
Alginate (*algD*)	28(100)	11(100)
Exotoxin A(*toxA*)	23(82)	11(100)
Exoenyme S(*exoS*)	7(25)	3(27)
Exoenyme U(*exoU*)	20(72)	8(73)
PilB protein (*pil B*)	15(54)	5(45)
Pyoverdin	16(57)	5(45)
Pyocyanin	12(43)	6(55)
Beta-hemolysis	28(100)	11(100)
BlaKPC	16(57)	16(57)
MBLs (VIM-2 and IMP-15)	Null	Null

*No significant relationship was found between any virulence gene and 11 MDR *P. aeruginosa* isolates

The present study shows that almost all patients with positive *P. aeruginosa* isolates had ear discharge (82.1%) and were already treated with antibiotics (75%) (Table **1**). The study also found MDR *P. aeruginosa* which accounted for 39% of the isolates including resistant to antipseudomonal drugs, while a recent study in Jordan by Al dawodeyah *et al*. [6] showed that MDR *P. aeruginosa* isolates accounted for 52.5% of all isolates from respiratory tract samples of hospitalized patients, and all were susceptible to colistin. While the present study has indicated that one out of 28 *P. aeruginosa* isolates was resistant to colistin (3%) (Table **3**). The detection of one *P. aeruginosa* isolate resistant to colistin is alarming, since this finding has not been previously reported in all recent studies carried out either in Jordan or other Arab neighboring countries among *P. aeruginosa* clinical isolates [6 - 8, 20, 23, 24].

Generally, the studies from this region demonstrated that *P. aeruginosa* isolates from the ear and other human infections were still moderately susceptible to antipseudomonal drugs such as piperacillin, piperacillin–tazobactam, ticarcillin-clavulanate and ceftazidime and imipenem [6, 8, 21, 24]. Recently, studies found that MDR *P. aeruginosa* clones can be associated with *MBLs* genes, mostly *VIM* and *IMP* types, which can be acquired by either chromosomal mutations or horizontal gene transfer. [25 - 26]. Therefore, studies investigated the incidence of *MBLs* and *BlaKPC* genes in clinical isolates of *P. aeruginosa.* It is highly important to select a proper treatment and to avoid the development of chronic cases.

The present study found that all 28 *P. aeruginosa* carried many potential virulence factors, including hemolysis and pigments as well as *las*B, *alg*D, *toxA*, *exoU*, and *pil B* genes in the range (100-54%), respectively (Table **4**), and this result is similar to the recently reported study about respiratory isolates in Jordan [6]. Other studies found high prevalence of *toxA*, *exoU*, and *pil B* genes in *P. aeruginosa* clinical isolates from other parts of the body [11, 27]. However, this study has not found any significant relationship (P=0.631) between MDR *P. aeruginosa* isolates and the presence of potential virulence genes.

CONCLUSION

In conclusion, this study has demonstrated that MDR *P.aeruginosa* isolates associated with certain virulence factors are important causative agents of otitis externa in Jordanian patients.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

The ethical approval was obtained from the Institutional Review Board (IRB) and the ethical Committee of the Jordan University Hospital, Jordan (2/2017, 10/1/2017).

HUMAN AND ANIMAL RIGHTS

Not Applicable.

CONSENT FOR PUBLICATION

Informed written consent was obtained from each participant.

AVAILABILITY OF DATA AND MATERIALS

The source of all clinical data of patients was obtained from their records at The Jordan University Hospital in Amman, and all sources of culture media, control bacterial strains, primers for virulence factors, potential resistance genes of ESBLs, KPC and MBLs are mentioned within text.

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CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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