Detection of Virulence Gene *ToxA* in *Pseudomonas aeruginosa* Isolated From Otitis Infection

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المستخلص

جمعت مئة وخمسين عينة من اصابات الأذن للكشف عن جين *tox A* في بكتريا *Pseudomonas aeruginosa* . زرعت هذه العينات على وسطي الماكونكي واكار الدم تحت ظروف معقمة. تم الكشف عن الخصائص المظهرية للعزلات واجراء الأختبارات الكيموحيوية في تشخيص بكتريا *P. aeruginosa* في هذه العينات ، وتم تأكيد التشخيص بواسطة بأستخدام VITEK- 2 . Compact system. من جميع هذه العينات تم الحصول على سبعة وعشرين عزلة تعود لبكتريا *P. aeruginosa* (18%) . اجري فحص الحساسية للمضادات الحيوية بأستخدامخمسة مضادات حيويةمن مجاميع مختلفة Ticarcillin 75 µg,Ceftazidime 30µg,Cefotaxime 30µg,Meropenem 10µg,Levofloxacin 5µg واختبرت حساسية جميع العزلات ضد هذه المضادات . بينت النتائج قدرة هذه العزلات على مقاومة اغلب المضادات . من بين هذه العزلات كانت هناك خمس عزلات (Q1, Q2, Q5, Q8 and Q11) اظهرت مقاومتها تجاه جميع المضادات . اختيرت هذه العزلات الخمسة المتعددة المقاومة للمضادات الحياتية للكشف عن جين *tox A* . وبعد استخلاص المادة الوراثية لهذه العزلات الخمسة بأستخدام كت خاص وتضخيم الجين *tox A* بأستخدام برايمرات خاصة ، كل هذه العزلات احتوت جين *tox A* بحجم مايقارب 535 زوج قاعدة في مادتها الوراثية .

Abstract

One hundred fifty swabs were collected from otitis infection to detect *toxA* gene in *Pseudomonas aeruginosa*. These samples were cultured on MacConkey agar and blood agar media under sterilization condition and incubated for 18-24 hrs at 37^{o}C. Morphological and biochemical test was carried out to identify *P. aeruginosa* in these samples , then diagnosis was confirmed with VITEK- 2 Compact system. In all these samples found twenty seven isolates belong to *P. aeruginosa* (18%).Antibiotic susceptibility test was achieved by select five antibiotics (Ticarcillin 75 µg,Ceftazidime 30µg,Cefotaxime 30µg,Meropenem 10µg,Levofloxacin 5µg) from different groups, all these twenty seven isolates were examined against these antibiotics. The results showed ability of these isolates to resist most antibiotics. Some of these isolates (Q1, Q2, Q5, Q8 and Q11)were resistant against all antibiotics. These five multidrug resistance isolates were selected to detect*tox A* gene. After extraction of genomeof all these five isolates by specific kit and amplificationof*tox A* gene by specific primers, all these five isolates were contained *toxA* gene in about 535 bp in its genetic materials.

Key words: *Tox A* gene, Otitis infection , Antibiogram.

Introduction

Otitis infection consider complex infectiousand inflammatory diseases in the infected ear and this infection increase in worldwide which infection found in about 80%in children.Otitis infection divide into twomajor kinds are acute infection and chronic infection [1,2]. Stage of acute infection include rapid clinical signs of inflammation, fever, bulging and possible puncture of the tympanic ear membrane .In absence of identification and treatment by physicians [3], acute infection progressing into chronic infection that include continuous drainage from the middle ear that linked with a perforated ear drum [4]and when examine by specialist physicians through otoscope may be seen chronic otitis infection signs include red and inflamed with purulent dischargein infected patients adults and children may led to hearing impairment[5,6]. Ear infection occur by different types of bacteria like *Pseudomonas aeruginosa* , *Streptococcus pneumoniae, Haemophilus influenzae,Moraxella catarrhalis,Klebsiella pneumonia* , *Staphylococcus aureus* and *Proteus vulgaris* in different countries in the world but include different causal agent and percentage of infection. *Pseudomonas aeruginosa* exhibit high levels of pathogenicity in different sites of the human body especially in otitis infection which it can increase biofilm formation that protect this bacterium from defenses of the human body especially macrophage and increase from it ability to resist antibiotics [7].*Pseudomonas aeruginosa* contain different virulence gene that encode to virulence factors as well as capacity of this bacterium to exhibit multidrug resistance because it has development mechanisms resistance against antibacterial [8].*P. aeruginosa* contain virulence genes that increase of pathogenicity like *tox A* gene that encoded into exotoxin A which lead to tissue necrosis through inhibition of protein synthesis in host cells and then will increase levels of infection and distribution of bacterial cell in tissue infecteted ear [9,10]. This study was aimed intodetection of virulence gene *ToxA* in *Pseudomonas aeruginosa* isolated from otitis infection.

Materials and methods

Collection of samples

From otitis patients infections, one hundred fifty specimens were collected in some hospitals in Baghdad during the period from September into December 2017. All samples prepared to the further experiment.

Laboratory diagnosis

Cultured all one hundred fiftyswabs from otitis infectiononto blood agar and MacConkey agar under sterilization condition and incubated for 18-24 hrs at 37^{o}C. Laboratory diagnosis was carried out by by their morphological characteristics and standard biochemical tests [11]. Then confirmation of *P. aeruginosa* by VITEK- 2 Compact system according to manufactures' instructions (Biomerieux/ France).

Antibiotic susceptibility test

Antibiotics included Cefotaxime 30µg (cephalosporins group),Ceftazidime 30µg (cephalosporin sgroup), Levofloxacin 5µg (flouroquinolones group),Meropenem 10µg (carbapenems group),Ticarcillin 75 µg (penicillins group). The results were compared with National Committee for Clinical Laboratory Standard (CLSI, 2014) [12].

Extraction of genetic material

Selected five multidrug resistance isolates (more resistant isolates) of *P. aeruginosa* to further experiments. Genetic material of these multidrug resistance was extracted by wizard® genomic DNA purification kit (Promega , USA) according to manufacturers' instructions. Amplification process of the *toxA* gene was carried out by using specific primers (table 1).

Table (1): Sequence primers of amplification *Tox A* gene

Primer type	Sequence 5'→3'	Expected amplicon size	Reference
Forward	CGAACTGGACGGTGGAGC	535bp	Azhar, 2017 [13]
Reverse	CCTGTTCCTTGTCGGGGATG		

Conditions of PCR cycleincluded Initial denaturation at 95 ºC for 1 minutes, 30 cycles of denaturation at 95ºC for 30 second, annealing at 57.2ºC for 30 second and extension at 72ºC for 1 minutes.Final extension at72ºC for 5 minutes.

Gel electrophoresis

PCR products of five multidrug resistance isolates were analyzed on agarose gel (1%) using horizontalelectrophoresis unit, gel was immersed in 0.5X TBE buffer, then samples were loaded into the wells of the gel . Electrophoresis was carried out for one - two hours at 50V. Then electrophoresis gel was stained with 10µl of ethedium bromide stock solution . DNA bands were visualized by using U.V transilluminator at 365 nm.and used DNA ladder (1500bp, Promega USA) [14] .

Results and Discussion

Laboratory diagnosis

Laboratory diagnosis focused on morphological and biochemical tests.Then chose bacterial isolate that non fermentatative of lactose on MacConkey agar . Results showed the isolates were Gram stain negative, oxidase positive, citrate positive , urea hydrolysis positive, catalase positive, bluish green pigmentation positive, indole production negative, voges proskaeur negative and methyl red negative . Then confirmed laboratory diagnosis of *Pseudomonas aeruginosa* by using VITEK- 2 Compact system according to manufactures' instructions (Biomerieux/ France). The results showed twenty seven of *Pseudomonosa aeruginosa* were obtained from all these otitis infection samples (18%).Isolation of *Pseudomonas aeruginosa*from otitis infection in this study is agreement with Ban *et al*.(2014)[15] in Baghdad and Zina, 2015[16] in Al-Hillah city. But it differs with other studies included Ali *et al*.(2013) [17] (9.2%) in Al-Hillah city , Ekram and Rokan.(2014) [18] (10%) in Kalar city,Rana *et al*.(2017) [19] (28%) and Ragiv *et al*.(2017) [20] (33%) in india. Thedifference in distribution of these bacteria may be belong to difference of geographical location.*Pseudomonas aeruginosa* consider most common

bacterium that causeotitis infection acute and chronic infection which consider chronic suppurative otitis media as a common ear diseases. Acute otitis media and otitis media with effusion are closely related clinical conditions [21,22]. Acute infection represents an acute infective process, otitis media with effusion include middle ear effusion in the absence of symptoms and signs of acute inflammation . *P. aerginosa* infection are the most causes otitis media infection. This bacterium contain different virulence gene encoded to virulence factors like tox A protein that necrosis host cells, also capacity of this bacterium to form biofilm that protect it from defenses of human body and antibiotics. Virulence gene expression of *P. aeruginosa* regulate by molecular signals called quorum sensing that include increase density of bacterial cells and increase produce these signals (chemical compounds) to trigger start infection with this dangerous bacterium [23,24].

Antibiotic susceptibility test

All twenty seven isolates exhibited different levels of it capacity resistance against antibiotics (table 1). The isolates (Q1, Q2, Q5, Q8 and Q11) resisted all antibiotics (100%) and chose as multidrug resistance to complete further experiment that include detection of *toxA* gene in these multidrug resistance isolates.Other *P. aeruginosa* isolates appeared resistance percentage (20% - 80%) against antibiotics, which most isolates were sensitive to meropenem (beta lactam group) that act on inhibition of cell wall synthesis.The difference in resistance may be due to randomly using of antibiotics , inappropriate duration lead to change level of antibiotic concentration in the body, insufficient dose and selection of effective antibiotic. These results agreement with Ragiv *et al*.(2017) [20] and Iara *et al*.(2017) [25]. Also it differs with Ali *et al*.(2013) [17] and Ekram and Rokan.(2014) [18].Increase density of this bacterium through quorum sensing signsls lead to increase virulence gene expression, then formation high level of pathogencity and high levels of resistance mechanisms. Mode of antibiotics action against *P. aeruginosa* may be inhibition of cell wall synthesis by Meropenem 10µg(carbapenems group)

and Ticarcillin 75 µg (penicillins group), inhibition of protein synthesis by Cefotaxime 30µg and Ceftazidime 30µg (cephalosporins group), inhibition of DNA gyrase activity in DNA replication by Levofloxacin 5µg (flouroquinolones group) [26]. Also *P. aeruginosa* posses different mechanisms of resistance these antibiotics like extended spectrum betalactamse production, modification of pores size in outer membrane , change of target site and efflux pump system that may be lead to multidrug resistance bacteria and formation clinical treatment problems in world because these multidrug resistance strains may be distribution through transfer genetic material or by mutation. Recently mechanisms of resistance increased in about 12 – 36% mostly in modification in outer membrane and efflux system as well as transport plasmid among them of *P. aeruginasa* isolates and then lead to difficult treatment [27].

Table (2): Antibiotic susceptibility test of *P. aeruginosa*isolates from otitis infection against five antibiotics

Isolates of *P. aeruginosa*	Cefotaxime 30µg	Ceftazidime 30µg	Levofloxacin 5µg	Meropenem 10µg	Ticarcillin 75 µg	Percentage of Resistance
Q1	resistance	resistance	resistance	resistance	resistance	100 %
Q2	resistance	resistance	resistance	resistance	resistance	100 %
Q3	sensitive	sensitive	resistance	sensitive	sensitive	20 %
Q4	resistance	sensitive	resistance	resistance	sensitive	60 %
Q5	resistance	resistance	resistance	resistance	resistance	100 %
Q6	resistance	sensitive	resistance	resistance	sensitive	60 %
Q7	resistance	resistance	sensitive	resistance	resistance	80 %
Q8	resistance	resistance	resistance	resistance	resistance	100%
Q9	resistance	sensitive	resistance	resistance	resistance	80 %
Q10	sensitive	resistance	resistance	sensitive	resistance	60 %
Q11	resistance	resistance	resistance	resistance	resistance	100 %
Q12	sensitive	resistance	sensitive	sensitive	resistance	40 %
Q13	resistance	resistance	resistance	sensitive	resistance	80 %
Q14	resistance	resistance	sensitive	sensitive	resistance	60 %
Q15	resistance	resistance	resistance	sensitive	resistance	80 %
Q16	resistance	sensitive	resistance	sensitive	resistance	60 %
Q17	resistance	resistance	sensitive	sensitive	resistance	60%
Q18	sensitive	resistance	resistance	sensitive	sensitive	40%
Q19	resistance	resistance	resistance	sensitive	resistance	80%
Q20	resistance	sensitive	sensitive	sensitive	resistance	%40
Q21	resistance	resistance	resistance	sensitive	resistance	80%
Q22	sensitive	resistance	resistance	sensitive	sensitive	40%
Q23	sensitive	resistance	resistance	sensitive	resistance	60%
Q24	resistance	resistance	sensitive	sensitive	resistance	60%
Q25	resistance	resistance	resistance	sensitive	sensitive	60%
Q26	resistance	resistance	resistance	sensitive	resistance	80%
Q27	sensitive	resistance	sensitive	sensitive	resistance	40%

Detection of *tox A* genein*P. aeruginosa* isolates

Antibiogram test was achieved of all twenty seven isolates of these bacteria , then depending on results of resistance the isolates (Q1, Q2, Q5, Q8 and Q11) exhibited ability to resist all antibiotics (multidrug resistance), so these five isolates were chose to detection of *tox A* gene. Genome of all these five isolates extracted by using specific kit , then amplification of *tox A* gene by specific primers. Product PCR of *tox A* was detected by gel electrophoresis on agarose against DNA ladder 1500bp (promga, USA) in about 535bp in all these five multidrug resistance isolates of *P. aeruginosa* as shown in figure 1,and these results agreement withAzhar, 2017 [13].

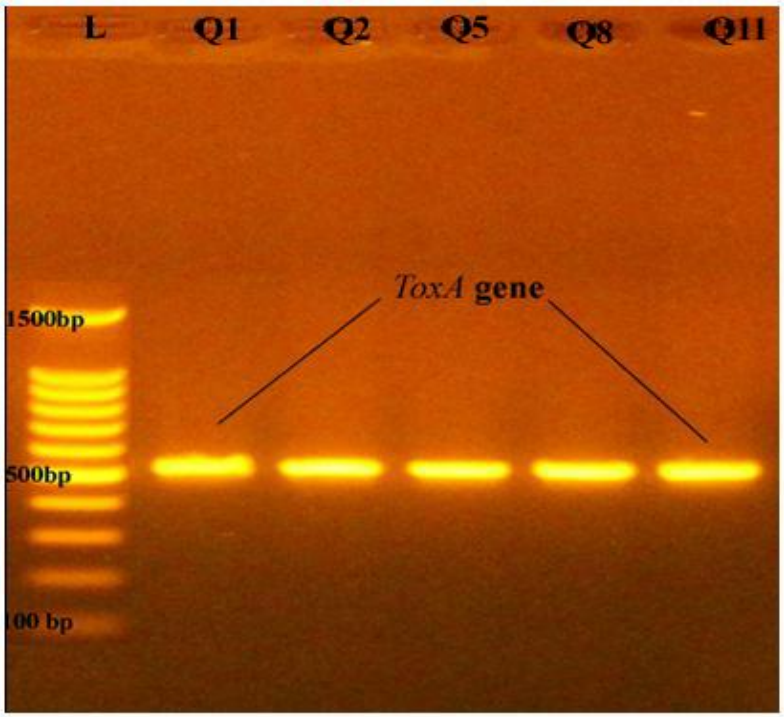

Figure (1): Gel electrophoresis for amplified *ToxA* gene inmultidrugresistance *P. aeruginosa* on agarose gel (1%) , 50V for 1 hour .

L: represent DNA ladder 1500bp.

Q1, Q2, Q5, Q8, Q11: Multidrug resistance*P.aeruginosa*isolates.

Conclusion

Otitis infection with *P. aeruginosa* from major clinical problems. Among 150 otitis infection found 27 isolates belong to *P. aeruginosa*(18%). These isolates were resistant to most antibiotics and

some of them (Q1, Q2, Q5, Q8 and Q11) isolates showed resistance against all antibiotics and all of these five multidrug resistance isolates were contained *tox A* gene in about 535 bp in its genetic material.

References:

1. **Asish, J.; Amar, M.; Vinay, H.; Avinash, S. S. and Amareshar, M. (2013).** To study the bacteriological and mycological profile of chronic suppurative otitis media patients and their antibiotic sensitivity pattern, Int J Pharma Bio Sci 4, 186–199.
2. **Aarhus, L.; Tambs, K.; Kvestad, E. and Engdahl, B. (2015).** Childhood otitis media: a cohort study with 30-year follow-up of hearing (The HUNT Study). Ear Hear 36, 302–308.
3. **Dickson, G. (2014). Acute otitis media. Prim Care 41, 11–18.**
4. **Qureishi, A.; Lee, Y.; Belfield, K.; Birchall, J. P. and Daniel, M. (2014).** Update on otitis media – prevention and treatment. Infect Drug Resist 7, 15–24.
5. **Yorgancilar, E.; Yildirim, M.; Gun, R.; Bakir, S.; Tekin, R.; Gocmez, C.; Meric, F. and Topcu, I. (2013).** Complications of chronic suppurative otitis media: a retrospective review. Eur Arch Otorhinolaryngol, 270, 69–76.
6. **Qureishi, A.; Lee, Y.; Belfield, K.; Birchall, J. P. and Daniel, M. (2014).** Update on otitis media – prevention and treatment. Infect Drug Resist 7, 15–24.
7. **Sun, J. and Sun, J. (2014).** Intracranial complications of chronic otitis media. Eur Arch Otorhinolaryngol, 271, 2923–2926.
8. **Li, J. D.; Hermansson, A.; Ryan, A. F.; Bakaletz, L. O.; Brown, S. D.; Cheeseman, M. T.; Juhn, S. K.; Jung, T. T. and Lim, D. J. (2013).** Panel 4: Recent advances in otitis media in molecular biology, biochemistry, genetics, and animal models, Otolaryngol Head Neck Surg 148, E52–E63.
9. **Luntz, M.; Yehudai, N.; Haifler, M.; Sigal, G. and Most, T. (2013).** Risk factors for sensorineural hearing loss in chronic otitis media. Acta Otolaryngol 133, 1173–1180.
10. **Tahir, M.; Jawaid, A.; Abdullah, A. and Najam, M.A. (2012).** Bacterial culture and sensitivity in active chronic otitis media: 500 cases in combined military hospital Rawalpindi, *Pak. J. Otolaryngol.*, 28: 56-8.
11. Harly , J. ; Prescott, L. (2002). Laboratory Exercises Microbiology. 5th

Ed. WCB. The Mcgraw- Hill, Companies.New York.

12. CLSI, (Clinical and Laboratory Standards Institute).(2014). Performance standard for antimicrobial susceptibility testing, Twenty-Fourth Informational Supplement, M100-S24.

13. Azhar, A.N.(2017). Molecular Detection of virulence factor genes in *pseudomonas aeruginosa* isolated from human and animals in Diwaniya province, Kufa Journal For Veterinary Medical Sciences , 8 (1):218-230.

14. Sambrook, J. and Russell, D.(2001). Molecular Cloning: Laboratory Manual. 3rd Ed. Cold Spring Harbor, New York.USA.

15. Ban, J. A.; Thanaa, R. A.; Waffa, A. A. (2014). Detection of *Exo* A Gene in *Pseudomonas aeruginosa* from Human and Dogs Using Polymerase Chain Reaction.Journal of Pharmacy and Biological Sciences, *9 (5): 38-41*.

16. Zina, B.A. (2015). Study of Bacterial Isolates and their Susceptibility Pattern in Chronic Suppurative Otitis Media.Journal of Pharmacy and Biological Sciences, *10 (6): 01-05.*

17. Ali, H. A.; Hayder, S.h.; Obayes, A.; Hussein , J. H.; Zahraa, M. and Sarah, K. Y. (2013). Otitis Media; Etiology and Antibiotics Susceptibility among Children under Ten Years Old in Hillah city,Iraq. Journal of Natural Sciences Research, 3 (3): 1-7.

18. Ekrem, K. and Rokan, D. K. (2014).Antibiotic susceptibility patterns of *Pseudomonas aeruginosa* strains isolated from various clinical specimens.Sky Journal of Microbiology Research, 2(2): 13 – 17.

19. Rana, M. A.; Arwa, M. A. and Abbas, F. A. (2017). Nucleotide sequences of the *Pseudomonas aeruginosa* algD gene isolated from Iraqi patients with otitis media. Current Research in Microbiology and Biotechnology, 5 (3): 1062-1070.

20. Rajiv, R. P.; Vijay, S.; Rajesh, K.; Kalyani, K. and Prabhat, K.(2017). Prevalence and Antibiotic Sensitivity of *Pseudomonas aeruginosa* Isolated from CSOM in NMCH, Patna, India. *Int.J.Curr.Microbiol.App.Sci* .*6*(6): 2912-2916.

21. Ali, Z.; Mumtaz, N.; Naz, S.A.; Jabeen, N. and Shafique, M.(2015). Multi-drug resistant *Pseudomonas aeruginosa*: a threat of nosocomial infections in tertiary care hospitals. J Pak Med Assoc. 65: 12-16.

22. Fahimeh, B. F.; Salehi, M.B.; Bahador, N. and Mobasherzadeh, S.(2015). Plasmid Mediated Antibiotic Resistance in Isolated Bacteria From Burned Patients Jundishapur. J Microbiol. 8(1): 13567.

23. Campos, C.; Gabarrus, A.; Liapikou, A.; Martinez, D.; Niederman, M. and Torres, A.(2016). Community-acquired pneumonia due to multidrug and non-multidrug resistant *Pseudomonas aeruginosa*. ERJ. 48 (5): 22-25.

24. Saleh, M.A. and Balboula, M.M.(2017). Plasmid Mediated Quinolone ResistanceDeterminants among Nosocomial Clinical *Pseudomonas aeruginosa* Isolates. IJCMAS. 6(1): 42-50.

25. Iara, R.; Gonc,A.; Raquel, C. D.; Melina, L. F.; Deivid, W.; Dafonseca, B.; Paulo, P. and Gontijo-Filho, R. M. (2017). Carbapenem-resistant *Pseudomonas aeruginosa*: association with virulence genes and biofilm formation.brazilian journal of microbiology , 48 (5) : 211–217.

26. Mansoor, K.; Tanvir, S.B.; Shariq, A.; Hussain, A.; Farooqi, B.J.; Ahmed, S. and Tanvir, R.(2015). Frequency and susceptibility pattern of Multidrug Resistant *Pseudomonas aeruginosa* in isolates of patients from a tertiary care hospital of Karachi, Pakistan. Eur J Biotechnol Bio Sci. 3(5):33- 36.

27. Tarig, M.S. and Eltayib, H.A.(2017).Multidrug Resistant Pseudomonas aeruginosa: Medical Impact, Pathogenicity, Resistance Mechanisms and Epidemiology. JSM Microbiology 5(3): 1046