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Molecular differentiation and determination of multi-drug resistant isolates of Pseudomonas species collected from burn patients in Kurdistan Region, Iraq

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Abstract

Background and objective: *Pseudomonas aeruginosa* is very a well-documented nosocomial and opportunistic microorganism, a little challenge is being present with the identification of such pathogen. This study aimed to identify Pseudomonas on genus and species levels by conventional PCR and determine multi-drug resistant isolates.

Methods: A total of 180 clinical isolates of *Pseudomonas species* were recovered from in and outpatients who attended Azadi and Rezgari Teaching hospitals in Duhok and Erbil city from October 2015 to May 2016. These isolates were phenotypically identified using standard microbiological procedures. A total of 100 isolates were randomly selected and confirmed at a molecular level as *Pseudomonas* spp.

Results: By applying genus-specific *gyr* B2 primer which produced1130bp amplification band and sixty-eight isolates were identified by PCR as *P. auerogino*sa using species-specific primer for 16S rRNA region which showed 956bpamplicon. Forty-six isolates out of the sixty-eight resembling *Pseudomonas aeruginosa* were diagnosed as being multi-drug resistant isolates by the disc diffusion method.

Conclusion: It can be concluded that multi-drug resistant isolates can pose a serious threat for the hospital-resident patients as increasing numbers of these isolates are being recorded in local settings.

Keywords: *Pseudomonas aeruginosa*; GurB2; Burn patients; Multi-drug resistance; *gyr* B2; Burn patients.

Introduction

Pseudomonas aeruginosa is very a well-documented nosocomial and opportunistic microorganism, and a little challenge is being present with the identification of such pathogen.[1] This opportunistic and intensely resistant microorganism exerts severe complications for hospitalized burn victims.[2] There are a number of standard procedures for identifying and diagnosing *Pseudomonas aeruginosa* based on bacterial culture methods eventually followed by basic morphological and biochemical differentiation. These ordinary methods are labor-intensive and time-consuming and usually not accurate enough to discriminate among genus and species.[3] Therefore, there is always a need to develop a rapid, specific, and sensitive method to detect the pathogen. The application of molecular techniques like polymerase chain reaction (PCR) assays for microbial identification and clinical analysis has come up with faster detection of pathogenic microorganisms.[4,5] Infections of *P. aeruginosa*, especially multidrug-resistant (MDR) isolates, in burn individuals, is usually proposed as a general complication. These victims are commonly at high risk for difficult-to-treat or untreatable infections.[6] Patients suffering from severe burns with immunological malfunction frequently manifest life-threatening situations, consequently, this nosocomial, opportunistic bacterium continues to create an important

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complication in burn-associated morbidity and mortality all over the world.[7] Multi-drug resistant (MDR) bacteria are defined as resistant to a minimum of three categories of antibiotics. Previous reports have shown that MDR gram-negative microorganisms result in increased mortality, extended durations of hospital admission and an elevated hospital costs when compared with conditions accompanying susceptible strains.[8] It is also documented that multidrug-resistant bacteria have usually been manifested as the etiology of nosocomial outbreaks in burn sections or as colonizers of the burn injuries.[9] This study aimed to identify *Pseudomonas* at the genus and species levels by molecular methods and determine the existence of multi-drug resistant isolates of *Pseudomonas aeruginosa* that can be recovered from burn lesions.

Methods

A total of 180 samples were collected from burn patients who attended Azadi and Rezgari Teaching hospitals in Duhok and Erbil cities, Kurdistan region, Iraq from October 2015 to May 2016; both sexes and all age groups were enrolled. All the samples were dealt with according to the standard microbiological procedures mentioned in the literature.[9] The enrolled isolates were subjected to DNA extraction experiments by the use of Wizard genomic DNA purification kits (Promega®, USA); the resulting DNA extract was visualized by UV light after being electrophoresed on 1% agarose gel.[10] Polymerase chain reaction was done by adding 1 ml of the bacterial DNA to 12.5 ml master mix (AccuPower PCR premix-® (South Korea)), then 1.2 ml (10 picomol\µl) of the genus-specific (*gyr* B2) primers[11] (Bioneer® (South Korea)) was also added, final volume was completed by adding 9.1 ml distilled water. Table 1 shows the primers sequences and amplification bands sizes of *gyr*B2 and 16 rRNA.[12] The PCR conditions for amplifying *gyr*B2 gene is illustrated in Table 2.

Table 1: Primers sequences and molecular weight of the relevant PCR products of *gyr*B2.

Gene	Forward primer	Reverse primer	Product size (bp)
*gyr*B2	5´TCCGGCGGTCTGCACGGCGT 3´	5´ TTGTCCGGGTTGTACTCGTC 3´	1130
16 sRNA	5´GGGGGATCTTCGGACCTCA 3´	5´TCCTTAGAGTGCCCACCCG 3´	956

Table 2: Cycling conditions of PCR - amplification of *gyr*B2 gene of Pseudomonas spp.

Step	Temperature(°C)	Time(minutes)	No. of Cycles
Initial denaturation	94	4	1
First loop:			
Denaturation	94	1	
Annealing	55	1	35
Extension	72	1	
Final extension	72	10	

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Species-specific primers were also used to amplify target 16 sRNA to confirm the identity of *Pseudomonas aeruginosa*. Two primers were used whose sequences are mentioned in Table 1. Preparations started with adding 4ml of the bacterial DNA to 12.5 ml master mix (AccuPower PCR premix-® (South Korea)), afterward, 1.0 ml (10 picomol\µl) of each of the species-specific primers (Bioneer® (South Korea)) was also added, final volume was completed to 25 ml by adding 6.5 ml distilled water. Table 3 describes the running conditions for the amplification of 16 sRNA gene of *Pseudomonas aeruginosa.*[13] The products of PCR experiments were run by electrophoresis on 2% agarose, DNA bands were visualized under UV light, and photographed by personal Camera.[10] The investigation of antibiotic sensitivity profile of *Pseudomonas aeruginosa* isolates proceeded by the application of disc diffusion method on Mueller-Hinton agar, and a group of antibiotics was carefully selected. Procedure strictly followed the guidelines of the National Committee for Clinical Laboratory Standards and Manual of Antimicrobial Susceptibility Testing.[14]

Results

The results of the current study showed that all the enrolled 180 isolates had shown phenotypic characteristics resembling those of *Pseudomonas spp*. The colonies produced special pigmentations and odor, they all were oxidase positive and yielded characteristic profiles on triple sugar iron medium and oxidation fermentation test distinctive of oxidative microorganisms. These test collectively followed the conventional methods used for the identification of Pseudomonas. The enrolled isolates also revealed the presence of bacterial genomic DNA after being subjected to a regular DNA extraction procedure. The results also confirmed that randomly selected one hundred isolates had been identified as *Pseudomonas spp*at the molecular level. (Figure 1)

Table 3: Running conditions for the 16 sRNA gene.

Step	Temperature(°C)	Time(minutes)	No. of Cycles
Initial denaturation	95	2	1
First loop:			
Denaturation	94	20 sec.	
Annealing	54	20 sec.	25
Extension	72	40 sec.	
Final extension	72	5	1

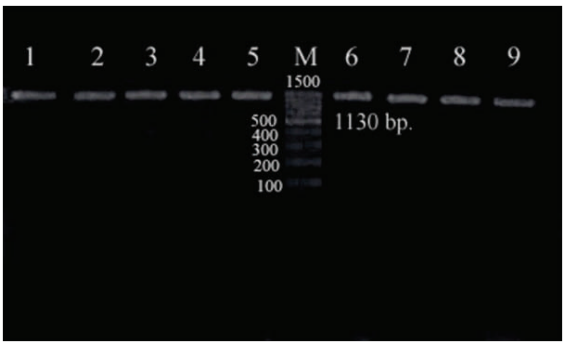

Figure 1: Species-specific PCR amplification for *Pseudomonas spp.* strains produced with gyrB2 amplicon with molecular weight 1130bp. Lane M contained DNA molecular weight marker (1500-100bp).

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For the molecular identification of *Pseudomonas aeruginosa*, the species-specific primer was used to detect target 16 S rRNA, and the result shows that sixty-eight out of the randomly selected one hundred isolates resulted in 956 bp species-specific amplicon (Figure 2). The existence of multi-drug resistant isolates has also been documented in the present work. It has been shown that forty-six isolates (nearly 68 %) out of a total of sixty-eight isolates of *Pseudomonas aeruginosa* revealed resistance to four major categories of antibiotics namely, Beta-lactams, aminoglycosides, monobactams, and folate pathway inhibitors. Thus, they were considered multi-drug resistant microorganisms. Interestingly, it was found that the most powerful antibiotics against this bacteria were the carbapenem (Imipenem) and the fourth generation cephalosporin (cefepime) which showed the lowest resistance percentages of 21% and 23%, respectively (Table 4).

Table 4: Antibiotic resistance profile shown by the studied isolates.

No	Antibiotic name	Category	Resistance No. (%) N = 68
1	Ticarcillin/clavulanic acid	Beta-Lactam	57 (83.8)
2	Ampicillin/Sulbactam	Beta-Lactam	58 (85.3)
3	Cefepime	Cephalosporin	16 (23.5)
4	Ceftazidime	Cephalosporin	32 (47.1)
5	Amikacin	Aminoglycoside	62 (91.2)
6	Tobramycin	Aminoglycoside	60 (88.2)
7	Ciprofloxacin	Fluoroquinolone	34 (50.0)
8	Meropenem	Carbapenem	25 (36.8)
9	Imipenem	Carbapenem	14 (20.6)
10	Aztreonam	Monobactam	63 (92.6)
11	Trimethoprim/Sulfamethoxazole	Folate pathway inhibitors	58 (85.3)

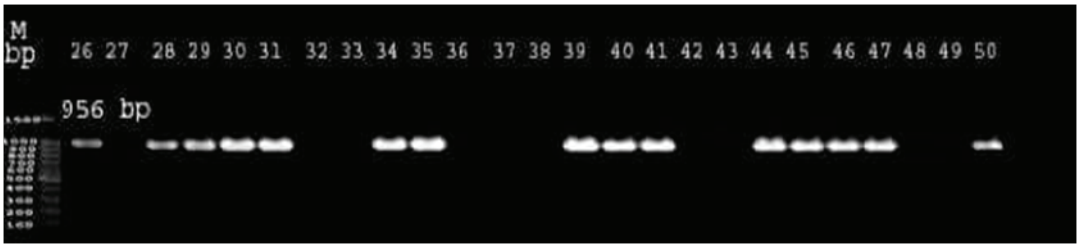

Figure 2: PCR product bands of the 16 S rRNA of *P. aeruginosa* on 1.5 % agarose gel. Lane M contains DNA ladder (1500-100bp).

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Discussion

Pseudomonas spp. has been documented as a usual pathogen responsible for a variety of human diseases and the expression of the diverse set of the gene is postulated as a determining factor for bacterial virulence.[15] *P. aeruginosa* is a greatly evolved nosocomial microorganism that is frequently encountered in hospital settings in part due to its ability to adhere to different surfaces through its polar pili and to minimize the cellular entry of antibacterial and antiseptics by the production of protective alginate. This pathogen also experiences extensive rearrangements of its chromosomal DNA which permit phenotypic conversion and thus increase the potential for developing antibiotics resistance and the capability to survive in aquatic conditions on limited nutrients supply.[16] The *gyr*B is a single-copy gene, found in all bacteria which codes for the ATPase domain of DNA gyrase, an enzyme plays essential role in the replication of DNA.[17] Earlier studies reported that gyrase subunit B2 gene contribute the major virulence properties of many bacterial species and has been used as a molecular tool for identification of bacterial species and phylogenetic analysis.[18]The results of the current study agreed with that of other who claimed that all collected isolates were positive for gyrB2 gene revealing the distribution and virulence properties of this gene in *Pseudomonas* species and confirm the detection of gyrB2 gene product of 1130bp using PCR as an identification marker.[12] Multidrug-resistant bacteria have commonly been reported as the cause of nosocomial outbreaks of infection in BUs or as colonizers of the wounds of burn patients. *Pseudomonas aeruginosa* has been demonstrated to be a leading cause of nosocomial infections in Iranian burn patients, and antimicrobial resistance has reached a critical point.[19] There has been rapid emergence of MDR *P. aeruginosa* in recent times, which is an important concern for clinicians who treat these infections. The results of the current study reported a much higher incidence rate of *Pseudomonas aeruginosa* than that reported by Kamara *et al*.,[20] and Navendu,[21] and higher incidence was also recognized by the present work when the result of Heggers and Good Heart[22] is taken into consideration. Interestingly, the current study obtained the lower percentage of resistance toceftazidime which did not match the results reported by Kamaria et al.,[20] and Indu,[23] and it was very far from the percentage reported by Srinivasan et al.[24] who stated that P. aeruginosa was 100% resistant to ceftazidime. Moreover, the present study showed a considerable effect for ciprofloxacin to which 50 % resistance appeared and this was inconsistent with the findings of Ashwin et al.[25] and Saha et al.[26] who mentioned that resistance to ciprofloxacin approached 90 %. It is worthy to say that the results appeared in the current work is comparable to those of Kamaria al[20] and Induet al.[23] when imipenem is considered and that all the studies obtained resistant isolates close to 20%. Collectively, MDR- *P. aeruginosa* (resistant to anti-microbial agents which are included in three or more anti-Pseudomonal categories (carbapenems, fluoroquinolones, penicillins / cephalosporins and aminoglycosides) with percentage exceeding 60 % observed in the present work constituted a challenging issue especially when lower percentages like that of Unanet al.[37] in Turkey, Sabir et al.,[28] in Pakistan, and Gad et al.[29]are compared. Importantly, some researchers favored the use of a combination of ceftazidime, amikacin, and sulbactam reduced the MICs of ceftazidime to the susceptible range in comparison with those of ceftazidime or amikacin alone, suggesting that this combination could be used against MDR *P. aeruginosa*. It has been well documented that colistin is the drug of choice for the treatment of pseudomonal infections but in a condition where it is not available for clinical use,

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combination therapy is highly recommended. Situations where the administration of colistin is minimal are those when the severe side effects like nephrotoxicity and neurotoxicity are appreciated and when safer alternatives are available.[30] This may explain the cause of the absence of colistin from the antibiotic list used in the current study. *Pseudomonas aeruginosa* has gradually become a major cause of nosocomial infections which occur in burn patients and which requires immediate and effective implementation of infection control strategies, to combat its spread. Rigorous monitoring for MDR among *Pseudomonas* isolates is very important because of outbreaks caused by strains which are resistant to potentially useful agents.[31]

Conclusion

It can be concluded that molecular methods can be rapid and powerful discriminating tools in terms of identifying microorganisms and that multi-drug resistant isolates of Pseudomonas aeruginosa pose a serious risk as they are distributed widely and score higher percentages with time.

Competing interests

The authors declare that they have no competing interests.

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