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Original Research

Distribution of bla_{TEM}, bla_{SHV}, bla_{CTX-M}, bla_{OXA}, and bla_{DHA} in *Proteus mirabilis* Isolated from Diabetic Foot Infections in Erbil, Iraq

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Abstract: Diabetic foot infection is considered to be one of the most important medical, economic, and social problems and a major cause of morbidity and mortality. *Proteus mirabilis* is a common etiologic agent of diabetic foot infections. This study aimed to determine the prevalence of beta-lactamase genes in *P. mirabilis* recovered from patients with diabetic foot wounds in Erbil, Iraq. Eighteen *P. mirabilis* isolated from 84 patients with diabetic foot ulcers were first phenotypically examined for the existence of extended-spectrum beta-lactamases by combined disc method and double-disc synergy method that all isolates showed positive results by both methods. The results were confirmed genetically by PCR to detect beta-lactamase-encoding genes (bla_{TEM}, bla_{SHV}, bla_{CTX-M}, bla_{OXA}, and bla_{DHA}). The results revealed that all isolates contained extended-spectrum beta-lactamase and that 80% of the *P. mirabilis* isolates contained bla_{DHA}, 60% had bla_{TEM}, 53.3% had bla_{OXA}, and 26.7% had bla_{CTX-M}, whereas no isolates harbored bla_{SHV}. The coexistence of two or more beta-lactamase genes in one isolate was observed. The existence of four genes (bla_{TEM} + bla_{CTX-M} + bla_{OXA} + bla_{DHA}) in the same isolate was documented in two isolates. In conclusion, this is the first study that reports a high prevalence of bla_{DHA} and the coexistence of four resistance genes in the same organism in *P. mirabilis* isolated from diabetic foot patients in Iraq.

Key words: Proteus; Diabetic foot; Beta-lactamase; Antibiotic.

Introduction

Infection of the foot is the most repeated and most feared complication in patients suffering from diabetes mellitus (1, 2) and the important cause of hospitalization related to diabetes (3). Infective agents are linked with very poor consequences, e.g., they can finally lead to the amputation of an infected foot, unless treatment approaches are immediately implemented (4, 5). Foot ulcers are linked with a higher risk of limb amputation in diabetic patients than in non-diabetic patients (6, 7).

The growing link between the multi-drug resistant (MDR) pathogens and the diabetic foot ulcers has complicated treatment strategies and increased the likelihood of amputation (8). MDR pathogens are responsible for long hospital stays, high management costs, and the morbidity and mortality in patients with diabetic foot infections (DFIs) (7). Many patients unnecessarily undergo amputations due to incorrect diagnosis and therapy (9). Knowledge about the pathogens causing infection in diabetic foot ulcers helps select ideal and effective antibiotic therapies (5, 10). However, the choice of therapies to be used relies on the accurate assessment of infection severity and dependable microbiologic data (11, 12). Although infections of the foot in patients with diabetes mellitus are first treated empirically, a treatment targeting the identified causative organisms may improve outcomes (13, 14). *Proteus* organisms sensitive to antibiotics are becoming resistant to drugs by producing beta-lactamases like extended-spectrum beta-lactamases (ESBLs) (15). *Proteus* species are part of the gram-negative bacilli involved in wounds, particularly in diabetic wounds (16). They cause serious infections, that are hard to eradicate, particularly in highly infected wounds (17). *Proteus* species that colonize the intestinal tract differ from those colonizing wounds in terms of their ability to possess antibiotic resistance genes (18).

Numerous studies have reported the prevalence of *Proteus* species in DFIs; however, data for drug resistance and production of ESBL in *Proteus mirabilis* isolated from DFIs are limited, particularly in Erbil, Iraq. Therefore, we investigated beta-lactamase production and prevalence of bla_{TEM}, bla_{SHV}, bla_{CTX-M}, bla_{OXA}, and bla_{DHA} genes in P. mirabilis isolated from diabetic foot patients in Erbil, Iraq.

Materials and Methods

Eighty-four diabetic patients that had foot ulcers admitted to hospitals from December 2016 to January 2018 were included in this study. Ethical approval was taken from the Institutional Ethical Committee of the College of Medicine/Hawler Medical University (paper code: 15) before conducting the study. Specimens were taken from patients after obtaining informed consent.

Inclusion criteria included any patients with diabetes and foot ulcers, those with an accidental diagnosis of diabetes after admission with a foot ulcer, and patients

with gangrene of the foot complicated by diabetes.

Patients were excluded if they were not willing to participate, had foot infections but were not diabetic, had gangrene of the foot with an etiology other than diabetes complication, had healed post-ulcerative site, pregnant or were under 18 years old.

Bacterial isolates

Eighty-four specimens collected from diabetic foot patients in Erbil, Iraq. We collected two specimens from the foot wound of each patient after cleaning the wound with gauze and normal saline, and then wound debrided by removing foreign material, necrotic tissue, calluses and damaged wound edges. No alcohol or iodine was used before specimen collection. Swabs were collected from the wound either by the Levine technique through rotation of the swab over a 1 cm^2 area for five seconds and using enough pressure to extract pus from the deep part of the wound or by aspirating purulent secretions using the syringe, or tissue biopsies were collected according to the wound type (19). The swabs were transferred into transport media (Sterile Swab with transport gel- LP ITALIANA / Italy) and taken to the Public Health Laboratory – Bacteriology Department for culture and identification. Specimens were cultured on Blood agar and MacConkey agar plates and then incubated at 37°C for 24 hours. Presumptive identification of *P. mirabilis* isolates was performed morphologically as having the swarming feature and non-lactose fermenting growth and then bacteriologically confirmed using Vitek2 identification system (BioMérieux Vitek, Marcy-l'Étoile- France) and the Vitek® 2 GN ID card (BioMérieux).

Antibiotic susceptibility testing

Susceptibility testing was done by using the Vitek2 System through the following manufacturer's instructions using a susceptibility test card (AST-GN69 TEST KIT-REF.413400) for gram-negative isolates**.**

Phenotypic identification of ESBLs

Double disc synergy test and combined disc methods were used to identify ESBL positive isolates.

Double disc synergy test

This test was performed using a combination of amoxicillin/clavulanate (AMC) with cefotaxime (CTX) and ceftazidime (CAZ) (Bioanalyse-Turkey) to detect ESBLs production according to the Clinical and Laboratory Standards Institute (CLSI) criteria for ESBL screening analysis (20).

The turbidity of prepared bacterial suspension was adjusted to 0.5 McFarland standards by using Densi-CHEK reader (BioMérieux), after which the samples were spread onto plates containing Mueller-Hinton agar media. An AMC disc (20 µg amoxicillin + 10 µg clavulanic acid) was placed on Mueller-Hinton agar plates; discs of 30 µg CAZ and 30 µg CTX were placed close to the first disc at a distance of 16–20 mm from the AMC disc, and incubated at 37°C for 24 hours. The organisms were considered ESBL producers once the inhibition zone around any of the cephalosporin discs showed an apparent increase towards the AMC disc.

Combined disc method

Isolates were tested using the combined disc method to detect ESBLs, with 30 µg CAZ/ 30 µg CAZ plus clavulanic acid 10 µg (CZC) discs and 30 µg CTX/ 30 µg CTX plus clavulanic acid 10 µg (CTC) discs (Bioanalyse, Ankara-Turkey). Tests were performed on Mueller-Hinton agar media. A difference of ≥5 mm between the inhibition zones of the CZC and CAZ, or between zones of the CTC and CTX, was considered to indicate the production of ESBLs as recommended by CLSI criteria (20).

Extraction of DNA

DNA of 15 *P. mirabilis* isolates was extracted by EzWay™ Genomic DNA Kit (bacterial) (KOMABIOTECH, Seoul, Korea). The gram-negative bacteria extraction protocol was used by following the manufacturer's protocol. The purity of extracted DNA was estimated by the Nano-drop system (Jencons Scientific Ltd., Leighton Buzzard, UK). The extracted genomic DNA was then confirmed by gel electrophoresis in a 1% agarose gel after staining with safe dye and visualized under a UV transilluminator documentation system. Suspensions containing DNA were stored at -20 °C until their use as a template for PCR (21, 22).

Molecular identification of *P. mirabilis*

Two primers were used to target the *16S rRNA* and *ureR* genes in the bacteria; those for 16S rRNA were designed to amplify 432 base pairs (bp) of 16S rRNA. The forward and reverse primer sequences and amplification conditions for both genes are shown in Table 1.

Table 1. Target genes, sequences of primers, Amplicon length, and cycling conditions of *16S rRNA* and *ureR* genes.

Primer Name	Primer Sequences (5' – 3')	Amplicon length (bp)	Conditions	Ref.
16S rRNA-F	CAT ATG GGA TTA GCT AGTAGG TGG GG	432	95 °C for 5 min 30 cycles: 95 °C for 30 s 60 °C for 30 s 72 °C for 30 s	Designed by authors
16S rRNA-R	CTC TAC AAG ACT CTA GCC AAC CAG			
ureR-F	GGT GAG ATT TGT ATT AAT GG	225	72 °C for 5 min 94 °C for 4 min 30 cycles: 94 °C for 40 s 58 °C for 1 min 72 °C for 20 s 72 °C 10 min	(23)
ureR-R	ATA ATC TGG AAG ATG ACG AG			

Table 2. Primers sequence and size used for amplification of beta- lactamase genes

Gene primer name	Nucleotide Sequences	Amplicon length (bp)	References
TEM-F TEM-R	5′-AGT GCT GCC ATA ACC ATG AGT G-3′ 5′-CTG ACT CCC CGT CGT GTA GTA A- 3′	431	(24)
SHV-F SHV-R	5′-GAT GAA CGC TTT CCC ATG ATG -3′ 5′-CGC TGT TAT CGC TCA TGG TAA-3′	214	(24)
CTX-M-F CTX-M-R	5′-CGC TTT GCG ATG TGC AC-3′ 5′-ACC GCG ATA TCG TTG GT-3′	550	(25)
OXA-F OXA-R	5′-ATT ATC TAC AGC AGC GCC AGT G-3′ 5′-TGC ATC CAC GTC TTT GGT G-3′	296	(24)
DHA-F DHA-R	5′-GTG GTG GAC AGC ACC ATT AAA-3′ 5′-CCT GCG GTA TAG GTA GCC AGA T-3′	314	(24)

Detection of beta-lactamase encoding genes

The following beta-lactamase encoding genes: bla_{TEM}, bla_{SHV}, bla_{CTX-M}, bla_{OXA}, and bla_{DHA} were screened. Forward and reverse primers were provided by Macrogen Company (Seoul, South Korea). Descriptions and sequences of these primers are displayed in Table 2.

Data analysis

Descriptive statistics were analyzed with SPSS version 23.0 software (SPSS, Inc., Chicago, IL, USA).

Table 3. Resistance of *Proteus mirabilis* to different antibiotics.

Antibiotic	*P. mirabilis* resistance	
	Frequency	%
Gentamicin	9	50.0
Tobramycin	9	50.0
Ciprofloxacin	7	38.8
Levofloxacin	11	61.1
Nitrofurantoin	18	100
Trimethoprim/sulfamethoxazole	15	83.3
Ampicillin	15	83.3
Amoxicillin/clavulanic acid	8	44.4
Ampicillin/sulbactam	10	55.5
Piperacillin/Tazobactam	3	16.6
Cefazolin	11	61.1
Ceftazidime	9	50.0
Ceftriaxone	9	50.0
Cefepime	9	50.0
Ertapenem	3	16.6
Imipenem	4	22.2

Results

Identification and antimicrobial susceptibility testing

Eighteen *P. mirabilis* isolates were isolated from 84 diabetic patients with diabetic foot ulcers, identified morphologically by swarming features and non-lactose fermentation growth, and confirmed to be *P. mirabilis* strains using the Vitek2 identification system. Antimicrobial susceptibility testing revealed a high prevalence of resistance to different antibiotics: all *P. mirabilis* isolates were resistant to nitrofurantoin. Resistance to trimethoprim/sulfamethoxazole and ampicillin was observed in 83.3% of strains; 61.1% of the isolates were resistant to levofloxacin and cefazolin, and 50% were resistant to gentamicin, tobramycin, ceftazidime, ceftriaxone, and cefepime, less resistance observed to piperacillin/tazobactam and ertapenem (16.7%). None of the *P. mirabilis* isolates were sensitive to all antibiotics. Most *P. mirabilis* isolates were resistant to three or more antibiotic classes (72.2%), which mean they were MDR as illustrated in Tables 3 and 4.

Molecular confirmation of *P. mirabilis* identity

Fifteen isolates were subjected to molecular identification by detecting the *16S rRNA* gene and urease production gene *ureR*. All isolates were confirmed as *P. mirabilis* except for one isolate, which could not be confirmed to contain both genes. Figure 1 shows a 432 bp amplicon of *16S rRNA* and Figure 2 shows a 225 bp amplicon of the *ureR* gene following electrophoresis on

Table 4. Resistance to different antibiotic classes.

Antibiotic classes resistance	No. of bacteria
Two classes	
-Penicillins + Fluoroquinolones	1
-Penicillins + Sulphonamide	1
-Fluoroquinolones + Sulphonamide	1
Three classes	
- Penicillins +Fluoroquinolones + Sulphonamide	1
Four classes	
- Penicillins + Cephalosporin + Sulphonamide +Aminoglycosides	1
-Penicillins + Fluoroquinolones + Sulphonamide + Carbapenems	1
Five classes	
-Penicillins + Cephalosporin + Fluoroquinolones + Sulphonamide +Aminoglycoside	6
-Penicillins + Cephalosporin + Fluoroquinolones + Sulphonamide + Carbapenems	2
Six classes	
Penicillins + Cephalosporin + Fluoroquinolones + Sulphonamide +Aminoglycoside + Carbapenems	2

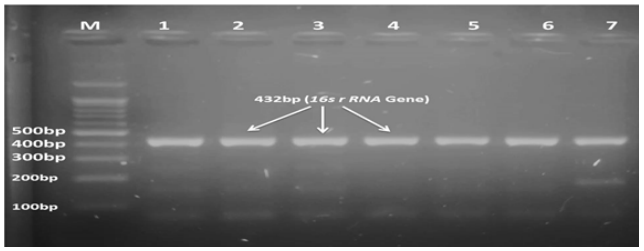

Figure 1. Gel electrophoresis of PCR product of *16S rRNA* of *Proteus mirabilis* (amplicon with 432 bp). Lane M: Ladder (100 bp); Lanes 1–7: Amplicon of *16S rRNA* of *P. mirabilis.*

an agarose gel stained with safe dye.

Detection of beta-lactamase genes

Phenotypic analysis of ESBLs identified all *P. mirabilis* isolates as ESBL producing, and confirmation at the molecular level showed that 80% of *P. mirabilis* isolates harbored bla_{DHA} and that 60% were bla_{TEM} positive, 53.3% were bla_{OXA} positive, and 26.7% bla_{CTX-M} positive. The bla_{DHA} gene was, therefore, the most detected gene in this study. No amplification products were obtained for bla_{SHV}.

Another important finding was that 66.6% of isolates contained two or more *bla* genes, even two strains were found to harbor four genes at the same time. The most common combination was bla_{TEM} + bla_{OXA} + bla_{DHA} (20%). Only one isolate that tested positive for ESBLs in the phenotypic tests was negative in the PCR analysis. As shown in Table 5 and Figures 3–5.

Discussion

High incidence of DFIs and amputation between diabetic patients has become an important public health issue in developing and developed countries. P. mirabilis is considered an important cause of foot wound infections in patients with diabetes (2, 26, 27). P. mirabilis infection Treatment has now become complicated in DFIs because of the acquired resistance to a large number of prescribed antibiotics in hospital settings. ESBL detection is not routinely performed in clinical laboratories in Iraq. Therefore, the emergence of ESBLs has become an important issue in the treatment of diabetic

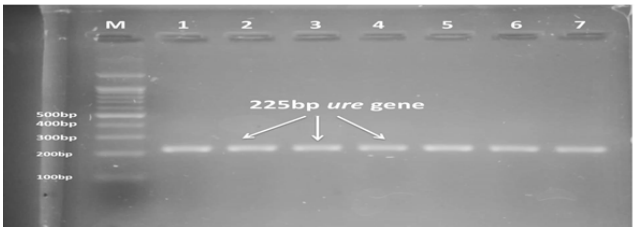

Figure 2. Gel electrophoresis of PCR product of *ureR* gene of *Proteus mirabilis* (amplicon with 225 bp). Lane M: Ladder (100 bp); Lanes 1–7: Amplicon of *ureR* of *P. mirabilis.*

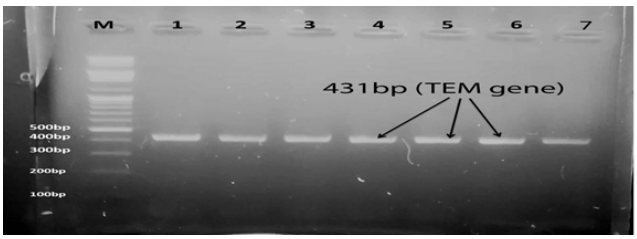

Figure 3. Detection of the gene encoding bla_{TEM} in ESBL-producing Proteus mirabilis. Lane M: Ladder (100 bp); Lanes 1–7: The 431 bp PCR product of bla_{TEM}.

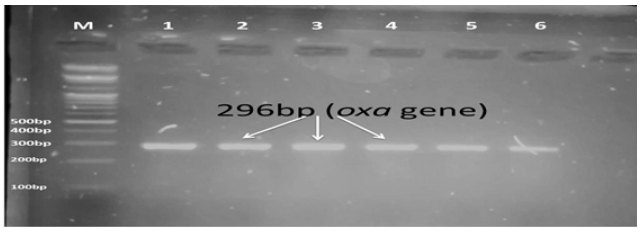

Figure 4. Detection of the gene encoding bla_{OXA} in ESBL-producing Proteus mirabilis. Lane M: Ladder (100 bp); Lanes 1–6: The 296 bp PCR product of bla_{OXA}.

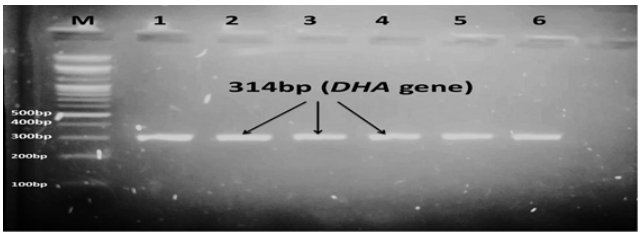

Figure 5. Detection of gene encoding bla_{DHA} AmpC beta-lactamase in Proteus mirabilis. Lane M: Ladder (100 bp); Lanes 1–6: The 314 bp PCR product of bla_{DHA}.

Table 5. The *bla* genes detected in *Proteus mirabilis* isolates.

***Proteus mirabilis* positive by PCR for beta-lactamases genes**	**Positive**	
A. Single beta-lactamases gene	**Number**	**%**
bla_{TEM}	9	60.0
bla_{SHV}	0	0.0
bla_{CTX-M}	4	26.7
bla_{OXA}	8	53.3
bla_{DHA}	12	80.0
B. Two or more beta-lactamase genes		
bla_{TEM} + bla_{DHA}	1	6.6
bla_{OXA} + bla_{DHA}	1	6.6
bla_{TEM} + bla_{OXA}	1	6.6
bla_{TEM} + bla_{CTX-M} + bla_{DHA}	2	13.3
bla_{TEM} + bla_{OXA} + bla_{DHA}	3	20.0
bla_{TEM} + bla_{CTX-M} + bla_{OXA} + bla_{DHA}	2	13.3

foot patients in Erbil, Iraq.

MDR organisms are predominantly isolated from wounds of diabetic foot patients compared to non-diabetic patients, which lead to the failure of empirical treatments and increased mortality and morbidity (28). A very high rate of antibiotic resistance was observed in this study, this result agrees with Datta et al. (29) and Pal et al. (30) results. The treatment of choice remains carbapenem or a combination of beta-lactam/beta-lactamase inhibitors as mentioned elsewhere (31); however, in this study, resistance to these antibiotics was observed, with 22.2% of the microorganisms being resistant to imipenem and 16.6% being resistant to ertapenem (both are related to the carbapenem group); 55.5% were resistant to ampicillin/sulbactam, 44.4% to amoxicillin/clavulanic acid, and 16.6% to piperacillin/tazobactam (the combination of beta-lactam/beta-lactamase inhibitor). The same result was gained by Adeyemo et al. (32). These data raise concerns regarding the available treatment options that must be considered by clinicians.

Antibiotic resistance among DFIs remains a major problem; it worsens the prognosis and may lead to poor treatment outcomes. This occurs largely because of the inappropriate use of antibiotics and free access to antibiotics in many countries. Thus, treating physicians must identify the presence of MDR organisms and their associated risk factors, like hospitalization, irrational use of antibiotics, chronic wounds and duration of diabetes, to improve outcomes, as stated by Data et al. [29] and Anvarinejad et al. (29, 33). Most patients had suffered from diabetes for a long time, and their foot ulcers remained unhealed for long time, e.g. some remain unhealed for ~2 years. Therefore, the widespread use of antimicrobial agents exerts selective pressure on the resistance of organisms.

The results showed that all *P. mirabilis* isolates were phenotypically ESBL producers, and none of them was sensitive to all tested antibiotics. This very high incidence of ESBLs in *P. mirabilis* was higher than that reported by Chaudhry et al. (34)] and Chen et al. (35). This may be because of differences in the types of samples, time of sample collection, and duration of antibiotic administration to patients.

ESBL producing *P. mirabilis* isolates are commonly observed to be MDR because of the presence of multi-resistant genes. In this study, our isolates were not only resistant to the beta-lactam group, but also other antibiotic classes such as aminoglycosides, sulphonamides, and fluoroquinolones; this pattern of resistance is similar to that described by Alabi et al. (36).

In this study, various beta-lactamase genes detected among the P. mirabilis isolates $bla_{TEM,}$ bla_{SHV}, bla_{CTX-M}, bla_{OXA}, and bla_{DHA}. According to the results, bla_{DHA} was the most prevalent type (80%), followed by bla_{TEM} (60%) and bla_{OXA} (53%). One isolate was genetically negative to all tested beta-lactamase genes, although it was phenotypically positive. This may be because other beta-lactamase genes were not evaluated in this study. The high occurrence of bla_{DHA} in this study was inconsistent with two other studies performed in Iran and Egypt, in which no *P. mirabilis* isolates were positive for bla_{DHA} screening (37), (38). However, in a study in Taiwan, bla_{TEM} was the most common resistance gene (39). In two other studies conducted in Iraq and Korea, the most dominant gene was bla_{CTX-M} (40, 41). Reports of bla_{DHA}-producing organisms are relatively rare, and the detection of bla_{DHA} in *P. mirabilis* was first reported in France (42) and then in Korea (43). This is the first study to identify bla_{DHA} among P. mirabilis isolated from diabetic foot patients in Erbil, Iraq. The emergence of bla_{DHA} has raised concerns because of its association with higher mortality rates, as mentioned by Fam et al. (44) and Park et al. (45).

The coexistence of different beta-lactamase genes within the same isolates was reported by Ibrahimagić et al. (46)] and Pokhrel et al. (47). The results of the present study showed that most *P. mirabilis* isolates contained two or more beta-lactamase genes. Among the coexisting beta-lactamases genes, $bla_{TEM} + bla_{OXA} + bla_{DHA}$ were the most common (20%). The most noticeable coexistence was observed in two isolates (13.3%) that harbored four resistance genes encoding $bla_{TEM} + bla_{CTX-M} + bla_{OXA} + bla_{DHA}$, and were phenotypically resistant to five classes of antibiotics, penicillins, cephalosporins, aminoglycosides, fluoroquinolones, and sulphonamides. The coexistence of different beta-lactamase genes within the same isolates will greatly limit present and future therapeutic options.

P. mirabilis MDR prevalence was very high in diabetic foot patients in Erbil, Iraq. Most were resistant to three or more antibiotic classes, which may be a consequence of the indiscriminate use of antibiotics. The most common beta-lactamase gene was bla_{DHA}. The presence of more than one resistance gene in bacteria limits the choice of antibiotic treatment. Thus, understanding antibiotic resistance patterns is essential for formulating treatment plans for patients with diabetic foot ulcers by using suitable antibiotics. This step will reduce resistance patterns, and minimize healthcare costs.

Data availability

The data that support this study finding is available upon request from the corresponding author.

Conflicts of interest

No conflict of interest regarding the publication of this paper.

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