DETECTION OF PROTEUS SPECIES IN DIABETIC WOUNDS AND THEIR ANTIBIOTIC RESISTANCE PROFILE ANALYSIS

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This work was carried out to determine the incidence of Proteus species infection in patients had diabetic foot wounds admitted to Vascular Surgery Department at Assiut University Hospitals. Proteus isolates were the most isolated organism (37.73%) followed by Klebsiella spp. (22.64%), then Pseudomonas spp. (20.75%) and E. coli (18.87%). Proteus mirabilis was the most isolated species represented (78%) followed by Proteus vulgaris (13%) then Proteus penneri (9%).

The antimicrobial susceptibility patterns of the isolated Proteus spp. were determined using agar disk diffusion method. The highest sensitivity was to ertapenem 76 isolates (76%). The antibiotic sensitivity then decrease in descending manner to amikacin (65%) > meropenem (54%) > imipenem (52%) > cefipime (49%), while the highest resistance rates were to amoxicillin–clavulanic acid 75 isolates (75%) > co-trimoxazole (73%) > cefoxitin (63%) > ciprofloxacin (49%). Plasmid DNA profile analysis of 10 MDR Proteus mirabilis that were common resistant to ceftriaxone was studied. Plasmid bands of six resistant Proteus mirabilis isolates were shown at 800bp while the others at 700 bp.

Plasmid curing was done by EtBr and SDS. Results of plasmid curing using ethidium bromide sublethal concentration of EtBr 1.25% showed that 7 cured cells become sensitive to ceftriaxone (30 µg), while 3 non cured cell still resistant to ceftriaxone. Plasmid curing using SDS sublethal concentration of SDS 1.2% cured only one of ten Proteus mirabilis which become sensitive to ceftriaxone and lost its band at 800bp, the other Proteus mirabilis not cured by SDS.

INTRODUCTION

Diabetic wound lesions are a major medical, social and economic problem and are the leading cause of hospitalization for patients with diabetes1.

It is one of the world’s major important health complications as well as a significant factor in the cost of inpatient treatment, loss of lives, disability and a reduction in life expectancy2.

Diabetic wounds/or foot ulcers and infections can lead to amputation of the foot or leg and one out 15 diabetic patients requires a limb amputation during their lifetime3. According to Ravisekhar et al.1 several enterobacteria and Gram positive bacteria have been found to be associated with diabetic foot ulcers; therefore this should be a matter of great concern for those who treat and rehabilitate diabetic wounds.

Foot infections are the most common complications of diabetic foot and plays a main role in the development of moist gangrene4. Pseudomonas spp., Enterococcus spp. & Proteus spp. carry a special role and are responsible for continuing and extensive tissue destruction with the poor blood circulation of the foot5. Proteus colonizing the intestinal tract and wounds vary in their carriage of genes encoding antibiotic resistance6.
The routine use of antimicrobial agents in both human and veterinary medicine has resulted in widespread antibiotic resistance and the development of antibiotic resistance genes especially within and between the gram-negative bacteria. With the presence of antibiotics selective pressure, these resistant Proteus species tend to persist, enabling the organism to cause extra infections such as septicemia.

The increasing association of multidrug resistant organisms (MDROs) with diabetic foot ulcers increases the risk of limb amputation. Infection with MDROs is also responsible for the increased duration of hospitalization, cost of management, morbidity and mortality of the diabetic patients. Plasmids serve a central role in mechanisms of bacterial antibiotic resistance. Plasmid sometime can be eliminated or lost from host cells by various treatments. This process termed curing. Curing may occur spontaneously or induced. It is greatly increased by application of some physical and chemical factors such as acridine dye, sodium dodecyl sulfate (SDS) and ethidium bromide dye. Using of heavy metals, ultraviolet, ionizing radiation or growth at temperature above the optimum may also result in elimination of the plasmid.

Some commonly used curing treatments are acridine mutagens, ion and ionizing radiation, thyme starvation, antibiotics and growth above optimum temperature, pH or extreme environmental conditions.

Appropriate selection of antibiotics based on the antibiogram of the isolates from the lesions is most critical for the proper management of these infections. Nevertheless, the initial empirical therapy is often decided based on the knowledge of the susceptibility profile of the prevalent microbial flora recovered from the previous cases.

The magic bullets, the miraculous drugs, antibiotics can be used to heal the diabetic wounds and thus the complications, which are a threat to all diabetic patients and thus can be minimized to a great extent.

METHODS
Isolation
This study included 251 patients admitted to Vascular Surgery Department at Assiut University Hospitals. They were of different ages (30-82 years) and sex (141 males and 110 females). Samples were collected from patients with diabetes mellitus (type1 and type 2) had wound foot infection. Two hundred and fifty one different clinical samples were collected under aseptic condition. These samples included debridement material and drained pus obtained during surgery from lesions.

Isolation of gram negative bacteria including Proteus spp.
The specimens were collected with sterile swabs and inoculated on blood agar and MacConkey agar at 37°C for 24h. Identification of Proteus isolates and other Gram negative bacteria by conventional biochemical tests such as catalase test, oxidase test, nitrate reduction test, IMVC test, urease Christensen's test, triple sugar iron test (TSI), motility indole ornithine medium (MIO), maltose fermentation test and citrate test.

Antibiotic susceptibility test
Antimicrobial susceptibility testing of Proteus isolates by the Kirby-Bauer disc diffusion method according to Clinical and Laboratory Standards Institute. The antimicrobial agents tested were Amoxicillin-clavulanic acid (20/10 µg), Piperacillin (100 µg), Imipenem (10 µg), Meropenom (10 µg), Ertapenem(10 µg), Cefoxitin (30 µg), Ceftriaxone (30 µg), Cefipime (30 µg), Ciprofloxacin (5 µg), Levofloxacin (5 µg), Amikacin (30 µg), Co-triamoxazole (1.25/23.75 µg). inoculated plates and incubated overnight. The zones of inhibition were measured and interpreted.

Plasmid profile analysis
Ten multidrug resistant Proteus isolates which were resistant to at least three antibiotics agents of 3 different groups including ceftriaxone as common agent were selected for plasmid analysis.

Extraction of plasmid DNA was done following rapid alkaline method. DNA is separated by gel electrophoresis based on its molecular mass, the bands of sample is compared to the DNA ladder so can determine their approximate size.
Plasmid curing

The methods described by Trevors and Iwalokun et al. were used in this study to cure plasmids using two different agents;
1- Sodium dodecyl sulphate (SDS) at final concentrations as follows: (0.2, 0.4, 0.5, 0.8, 1, 1.2, 1.4, 1.6, 1.8 and 2%).
2- Ethidium bromide at final concentrations as follows: (0.75%, 1.25% and 1.5%).

The ten P. mirabilis isolates which their plasmid profile analysis previously determined were selected. After treatment of bacterial isolates with curing agent, colonies that still able to grow on nutrient agar were selected randomly and were replica plated on nutrient agar plates containing the antibiotic discs to which the wild isolate was resist. Plates then incubated at 37°C for 24 hrs to test sensitivity to ceftriaxone after curing. Plasmid extraction of cured strains and agarose gel electrophoresis with 1% agarose was done again.

RESULTS AND DISCUSSION

In the present study, the majority of diabetic wound infection were caused by mixed infection of 2 organisms (71.7%). The monomicrobial infection was (28.3%). In consistent with this work Raja reported that 42% of patients developed mixed growth. Similarly, Llanes et al. reported that 58.9% of cases were polymicrobial in nature. Other studies from Jamaica and France documented that the prevalence of polymicrobial infection could be as high as 80-87.2%. In this study, Proteus spp. was the most isolated organism (37.73%) followed by Klebsiella spp. (22.64%), Pseudomonas spp. (20.75%) and E. coli (18.87%). This was matching with the studies of Raja and Oguachuba which showed that Proteus spp. was the commonest gram-negative etiological agent from wound infections. Also Ramakant et al., who studied the changing of microbiological profile of pathogenic bacteria isolated from DFU in, Lucknow, India over a period of 8 years; 1632 cultures were isolated from 434 patients with diabetic foot infections, showing that Gram-negative bacterial infection was increasing from 50.6% to 66% and the most common isolates were P. aeruginosa, E. coli and Proteus spp.

In this research out of 251 clinical specimens, Proteus isolates were 100 which comprising 78 Proteus mirabilis (78%), 13 Proteus vulgaris (13%) and 9 Proteus penneri (9%) (Table 1). Mathew and Suchithra showed that the order of occurrence of gram negative isolates in diabetic wound ulcers was Proteus mirabilis (22.73%) > Enterobacter aeruginosa (18.18%) > Klebsiella pneumonia (18.18%) > Pseudomonas aeruginosa (13.64%) > Salmonella typhi (13.64%) > E.coli (9.09%) > Proteus vulgaris (4.55%).

Although P. mirabilis was isolated more frequently than the other Proteus species in this study (78%), however, it is lower than that claimed by Auwaerter (90%). Comparably to this research in Nigeria a total of 148 Proteus isolates comprising of 97 P. mirabilis and 51 Proteus vulgaris were isolated from diabetic wounds of diabetes patients attending Ahmadu Bello University Teaching Hospital Zaria, Kaduna State, Nigeria. In this research out of 251 clinical specimens, Proteus isolates were 100 which comprising 78 Proteus mirabilis (78%), 13 Proteus vulgaris (13%) and 9 Proteus penneri (9%) (Table 1). Mathew and Suchithra showed that the order of occurrence of gram negative isolates in diabetic wound ulcers was Proteus mirabilis (22.73%) > Enterobacter aeruginosa (18.18%) > Klebsiella pneumonia (18.18%) > Pseudomonas aeruginosa (13.64%) > Salmonella typhi (13.64%) > E.coli (9.09%) > Proteus vulgaris (4.55%).

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In the present study the majority of Proteus infection in diabetic wounds were detected in the age group >50-82 years which accounted for 45% of the positive cases. The infection rate of patient aged >40-50years was 32% followed by age group 30-40 years which was 23%. There was significant difference of the rate of Proteus infection among different age groups.

Concerning gender, sixty five patients (65%) were males while thirty five patients (35%) were females. The greater percentage of males in this study may be either due to males selectively presenting to health services or due to that males being more exposed to foot trauma in the outdoors. This is comparable to another study where out of 107 patients with diabetic foot from surgical units, 70 were males and 37 were females patients and the age ranged from 17 to 66 years with mean age being 43 years.

In the present study the antimicrobial susceptibility patterns of the isolated Proteus spp. were done using agar disk diffusion method. The highest sensitivity rate was to ertapenem 76 isolates (76%), amikacin (65%), meropenem (54%), imipenem (52%) and cefipime (49%) (Fig. 1). This partly coincide with study carried out by Makled and Alghamdi which showed that aminoglycosides commonly used in treatment of
**Table 1:** Identification of *Proteus Spp.*

| Test                                      | *Proteus mirabilis* | *Proteus vulgaris* | *Proteus penneri* |
|-------------------------------------------|---------------------|--------------------|-------------------|
| Indole production test                    | - ve                | + ve (red ring in alcoholic layer) | - ve              |
| Ornithine decarboxylase test              | + ve (purple color) | - ve (yellow color) | -ve               |
| Maltose fermentation test                 | - ve                | - ve               | +ve               |
| TSI tests                                 | Red slant, black butt | yellow slant, black butt | yellow slant, yellow butt |
| Citrate test                              | citrate positive    | citrate negative   | citrate negative  |
| No. of Proteus isolates (%)               | 78%                 | 13%                | 9%                |

**Fig. 1:** The antibiotic sensitivity test of *Proteus* isolates.
infections caused by *P. mirabilis* isolates are still effective. Also, the antimicrobial susceptibility testing carried out by El-Tahawy\(^3^0\) showed that imipenem was the most effective agent against gram-negative organisms.

The highest resistance rates in this work were to amoxicillin - clavulanic acid (75\%), co-trimoxazole (73\%), cefoxitin (63\%) and ciprofloxacin (49\%). Resistance of 77-85% of *Proteus spp.* against ampicillin, cotrimoxazole, tetracycline, and chloramphenicol was reported by Feglo et al.\(^3^1\) who added that the high level of β-lactamase production and multi-drug resistance of the isolates are indications of an increase in the resistance menace. In this study the resistance to ceftriaxone was (38\%), Similar results were reported by Jawad and Alramahy\(^3^2\) in which the resistant pattern of *Proteus spp.* to 3rd generation cephalosporins were to cefuroxime (37.1\%) and ceftriaxone (33.8\%).

Swenson Jana and Patel Jean\(^3^3\) showed that virtually all *Proteus vulgaris* and *Proteus penneri* strains are capable of producing inducible β-lactamases that will hydrolyze primary and extended-spectrum penicillin and cephalosporins. *Proteus mirabilis* which lacks intrinsic chromosomal β-lactamase genes is entirely dependent upon acquisition of different β-lactamase genes to express a β-lactamase-mediated resistance phenotype.

In the present study plasmid profile of 10 multidrug resistant *Proteus mirabilis* resistant to at least three antibiotics of 3 different groups including ceftriaxone antibiotic as the common agent was done. Ceftriaxone was chosen because it is the empirical treatment in Assiut University Hospitals. Ceftriaxone has been effective in treating infections due to MDR enterobacteriaceae where the long half-life of the drug result in worthwhile convenience and cost benefits\(^3^4\). It is a choice drug for surgical prophylaxis and treatment. It is on the WHO Model List of Essential Medicines (2016) as the most effective and safe medicinal needed in a health system\(^3^5\).

Plasmid DNA was obtained using alkaline lysis method\(^1^7\). It was found that all of the 10 multidrug resistant *Proteus mirabilis* had plasmid. The agarose gel electrophoresis for 6 of them showed bands at 800 bp and the other four showed at 700 bp.\(^2^7\) mentioned that the plasmid band of Proteus isolates showed at range from <0.45kb to >1.25kb (Fig. 2 & Table 2).

| Proteus species                  | Antibiotics resistance to antibiotic | Band bp |
|---------------------------------|-------------------------------------|---------|
| **Plasmid profile of 6 resistant Proteus mirabilis** | CRO, AMC, PRL, FOX, LEV, CIP CRO, FEP, AK, LEV, FOX CRO, AMC, LEV, CIP, FOX, MEM, FEP CRO, AMC, FOX, AK, MEM CRO, AMC, MEM, PRL, FEP CRO, AMC, FOX, FEP, PRL, CIP | 800     |
| **Plasmid profile of 4 resistant Proteus mirabilis** | CRO, AMC, AK, PRL, FEP CRO, AMC, AK, FOX, FEP CRO, AMC, AK, MEM, CIP CRO, AMC, FEP, FOX, PRL, CIP | 700     |

CRO (Ceftriaxone), AMC (Amoxicillin-clavulanic acid), AK (Amikacin), PRL (piperacillin), FOX (Cefoxitin), FEP (Cefipime), CIP (Ciprofloxacin), MEM (Meropenem), LEV (Levofloxacin).
In this work curing of plasmid was done by SDS and EtBr. 7 of *Proteus mirabilis* cured by EtBr lost their bands at either 800 bp or 700 bp became sensitive to ceftriaxone and the non-cured strains still resistant (Fig. 3).

The present study proved that EtBr with sublethal concentration at 1.25% was more effective as plasmid curing agent than SDS at 1.2% as it cured 7/10 of plasmids while SDS cured only one. The P value between the two reagents was significant (0.022) (Fig. 5).

Antimicrobial resistance in *Proteus* is of great public health concern in the developing world. The accelerated emergence of antibiotic resistance among the prevalence pathogens is the most serious threat on the management of infectious diseases.
Conclusion
1- The study concluded that Proteus infection of DFI represented 39.84% in Vascular Surgery Department of Assiut University Hospitals during the period of this study. This is considered high rate of infection.
2- There is high resistance rate of Proteus to many antibiotics mainly amoxicillin–clavulanic acid, co-trimoxazole, cefoxitin, and ciprofloxacin. So treatment of Proteus infection is problematic.
3- Ertapenem was the most effective antibiotics against Proteus. However, no single antibiotic was found to be an effective agent against all Proteus isolates.
4- Plasmid plays great role in Proteus resistance to ceftriaxone.

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Fig. 5: Comparison between Ethidium bromide and SDS used for curing plasmid of Proteus mirabilis.
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الكشف عن فصائل البروتينات المعزولة من عدوي جروح القدم السكري ونمط مقاومتها لمضادات الحياء

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تم هذا العمل لتحديد معدل الإصابة بعزلات البروتين في مرضى القدم السكري المترددين الذين تتراوح درجة الإصابة بين 100 و140 عينة، تم عزل عدوى نافع /C. albicans/ من صبغة الدم ونسبة 7,37%، إصابة إيجابية للعمر، لعدوى C. albicans من صبغة الدم ونسبة 62/62%، إصابة إيجابية للعمر، لعدوى H. influenzae من صبغة الدم ونسبة 18,72%، إصابة إيجابية للعمر، لعدوى H. influenzae من صبغة الدم ونسبة 18,72%.

تم دراسة عزل البروتينات للفصائل الحيوية تم استعمال طريقة الأجار المنتشر، وجد أن أكثر العزل حساسية للمضادات الحيوية هي تيلينيسين (62%) والهيدروكلوبازون (62%) ونسبة 54%، وإيجابية للفيسبينات (62%) ونسبة 49%، وإيجابية للفيسبينات (62%) ونسبة 49%، ووجد على مقاومة كانت ضد الأموكساسيلين - حمض الكلافولين بنسبة 73%، ونسبة (62%)، ونسبة 3%، ونسبة 3%، ونسبة 3%، ونسبة 3%.

عند دراسة عزل البروتينات من البروتينات ميبرابيلإت متعددة المقيدة بـ DNA البلازميدية، نجحت نتائج هذ التحدي بـ DNA البلازميدية في مقاومة البروتينات للفيسبينات لعقار السيفترياكسون، وجد أن الجذور البلازميدية موجودة في 6 عزلات من البروتين ميبرابيلإت مقاومة لعقار السيفترياكسون عند 800 ميبرابيلإت المحالة للفيسبينات السيفترياكسون ظهرت عند 700 ميبرابيلإت.

عوامل العزلة المحمولة على البلازميد بسداسي دوديسيل كبريتيت شمسي وائيديوم بروميدي في محاولة لتحليك محتواها البلازميدية. كانت النتيجة عند تحليك البلازميد باستخدام الائيديوم بروميدي عند تركيز 2,51%، 7 من العزلات اصبحت حساسة لعقار السيفترياكسون، بينما الـ 3 عزلات الأخرى مازالت مقاومة لهذا العقار. عند استعمال دوديسيل كبريتيت محلى بروميدي عند تركيز 1,2% وجد أن عزلة واحدة فقط من البروتين ميبرابيلإت اصبحت حساسة لعقار السيفترياكسون، بينما باقي العزلات لم يتم تحليك محتواها البلازميدية.