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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 mirabils 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 be 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 diabetes[1].

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 expectancy[2].

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 lifetime[3]. 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 gangrene[4], *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 foot[5]. *Proteus* colonizing the intestinal tract and wounds vary in their carriage of genes encoding antibiotic resistance[6].

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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[7]. With the presence of antibiotics selective pressure, these resistant Proteus species tend to persist, enabling the organism to cause extra infections such as septicemia[8].

The increasing association of multidrug resistant organisms (MDROs) with diabetic foot ulcers increases the risk of limb amputation[9]. Infection with MDROs is also responsible for the increased duration of hospitalization, cost of management, morbidity and mortality of the diabetic patients[10]. Plasmids serve a central role in mechanisms of bacterial antibiotic resistance[11]. 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[12].

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[13].

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[14].

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[15].

Antibiotic susceptibility test

Antimicrobial susceptibility testing of *Proteus* isolates by the Kirby-Bauer disc diffusion method according to Clinical and Laboratory Standards Institute[16]. 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[17]. 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[18] and Iwalokun *et al.*[19] 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[20] reported that 42% of patients developed mixed growth. Similarly, Llanes *et al.*[21], 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%[22]. 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[20] and Oguachuba[23] which showed that *Proteus spp.* was the commonest gram-negative etiological agent from wound infections. Also Ramakant *et al.*[24], 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[25] 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[26] (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[27].

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[28].

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[29] which showed that amino-glycosides commonly used in treatment of

Table 1: Identification of *Proteus Spp.*

	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%

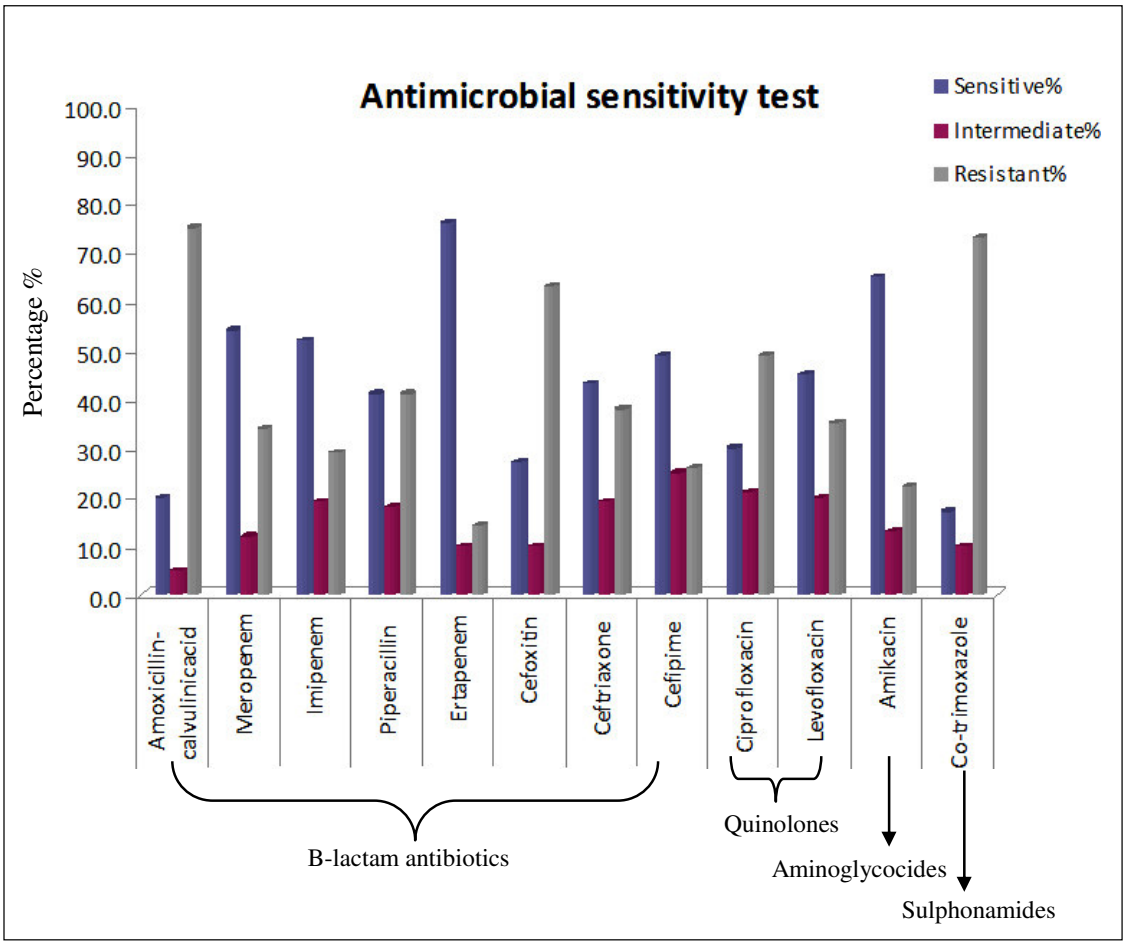

Fig. 1: The antibiotic sensitivity test of *Proteus* isolates.

infections caused by *P. mirabilis* isolates are still effective. Also, the antimicrobial susceptibility testing carried out by El-Tahawy[30] 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 isolates (75%), co-trimoxazole (73%), cefoxitin (63%) and ciprofloxacin (49%). Resistance of 77-85% of *Proteus spp*. against ampicillin, co-trimoxazole, tetracycline, and chloramphenicol was reported by Feglo *et al.*[31] 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[32] 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[33] 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 b-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[34]. 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[35].

Plasmid DNA was obtained using alkaline lysis method[17]. It was found that all of the10 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.[27] mentioned that the plasmid band of Proteus isolates showed at range from <0.45kb to >1.25kb (Fig. 2 & Table 2).

Table 2: Plasmid profile of ceftriaxone resistant *Proteus mirabilis.*

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).

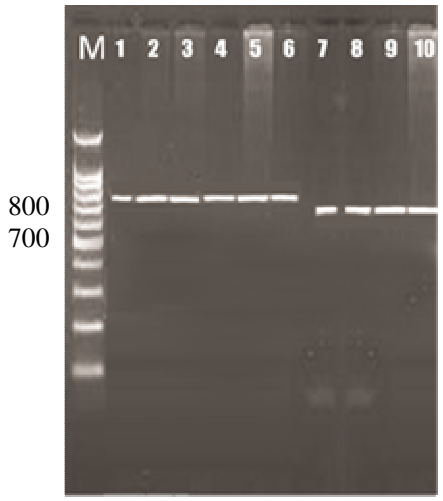

Fig. 2: Agarose gel electrophoresis of Plasmid DNA of *Proteus mirabilis* resistant to ceftriaxone. Lane M: DNA marker (100-1500) ladder, Lanes (1, 2, 3, 4, 5 and 6) show plasmid bands at 800 bp. Lanes (7, 8, 9 and 10) show plasmid bands at 700 bp.

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).

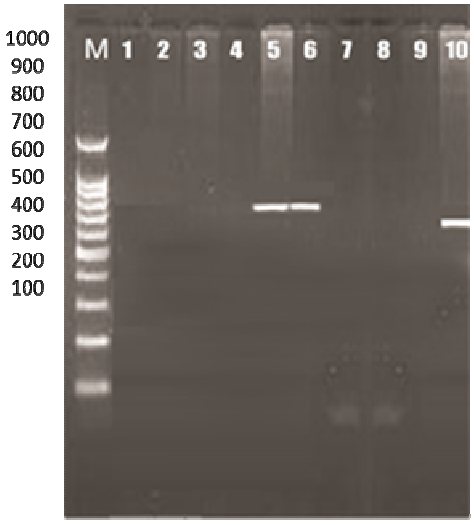

Fig. 3: Agarose gel electrophoresis of plasmid cured *Proteus mirabilis* by ethidium bromide. Cured *Proteus mirabilis* (1, 2, 3 and 4) lost plasmid bands at 800 bp. Cured *Proteus mirabilis* (7, 8 and 9) lost plasmid bands at 700 bp.

The same for the one strain cured with SDS became sensitive to ceftriaxone while the other 9 still resistant (Fig. 4). This proved that *Proteus* resistance to ceftriaxone was associated with plasmid had approximate sizes 700-800 bp. There is a possibility that these plasmids could carry other antibiotic resistance genes which didn't carried out in this study. Iwalokun *et al.*[19] showed that plasmid curing with 1.25% ethidium bromide solution in some of *Proteus Spp*. confirmed strongly the involvement of chromosome and plasmid in antibiotic resistance. But, the present result revealed that the β-lactamase genes mostly located on plasmids for the analyzed strains. This result was in agreement with the finding of other investigators[36] confirmed the location of antibiotic markers on R-plasmid by treating the cells with curing agents.

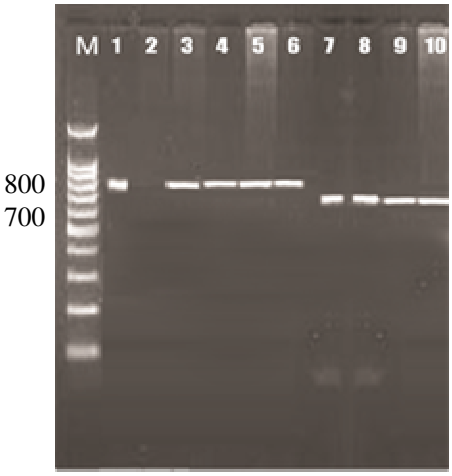

Fig. 4: Agarose gel electrophoresis of plasmid cured *Proteus mirabilis* by 10% SDS the cured cell lost its band (800 bp) on lane 2.

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.

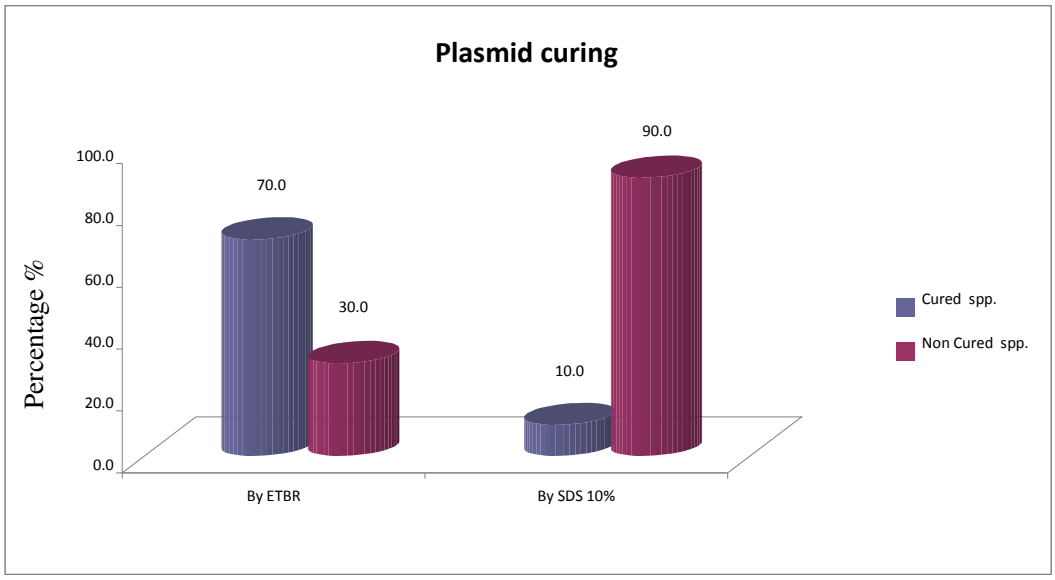

Fig. 5: Comparison between Ethidium bromide and SDS used for curing plasmid of *Proteus mirabilis*.

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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نشرة العلوم الصيدلية
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الكشف عن فصائل البروتيس المعزولة من عدوي جروح القدم السكري ونمط مقاومتها للمضادات الحيوية

اماني جمال ثابت – إحسان عبد الصبور – أماني محمد عدوي نافع – محمد أحمد المختار – يسرا عزت بيومي

قسم الميكروبيولوجيا الطبية والمناعه ، كلية الطب ، جامعه أسيوط ، أسيوط ، مصر

تم هذا العمل لتحديد معدل الاصابة بعزلات البروتيس في مرضي القدم السكري المترددين الي قسم جراحة الاوعية الدموية بمستشفي اسيوط الجامعي. كانت معظم العزلات من البروتيس في ١٠٠ عينة بنسبة (٣٧,٧٣%) يليها عزلات الكليبسيلا في ٦٠ عينة بنسبة (٢٢,٦٤%) ثم عزلات الزائفة الزنجارية في ٥٥ عينة بنسبة (٢٠,٧٥%) وعزلات الايكولاي في ٥٠ عينة بنسبة (١٨,٨٧%). البروتيس ميرابيليس ٧٨ عينة بنسبة (٧٨%) والبروتيس فولجاريس ١٣عينة بنسبة (١٣%) والبروتيس بينيري ٩ عينات بنسبة (٩%).

تم دراسة نمط عزلات البروتيس للمضادات الحيوية تم باستعمال طريقة الاجار المنتشر ، وجد ان اكثر العزلات حساسية للمضاد الحيوي ايرتابينيم بنسبة (٧٦%) يليه الاميكاسين (٦٥%) والميروبينيم (٥٤%) والإيميبينيم (٥٢%) والسيفيبيم (٤٩%) واخيرا الليفوفلوكساسين (٤٥%). ووجد اعلي مقاومة كانت ضد الاموكساسيللين- حمض الكلافيولينك بنسبة (٧٥%) يليه الكوتريموكسازول (٧٣%) والسيفوكستين(٦٣%) واخيرا السيبروفلوكساسين (٤٩%).

عند دراسة نمطDNA البلازميدي لعشر عزلات من البروتيس ميرابليس متعددة المقاومة للمضادات الحيوية خاصة لعقار السيفترايكسون وذلك لمعرفة دور DNA البلازميدي في مقاومة البروتيس ميرابليس لعقار السيفترايكسون. وجد انً الحزم البلازميدية موجودة في ٦ عزلات من البروتيس ميرابليس المقاومة لعقار السيفترايكسون عند ٨٠٠ bp ، بينما الاربع عزلات الاخري المقاومة للسيفترايكسون ظهرت عند ٧٠٠ bp.

عوملت العزلات المحتوية علي البلازميد بمادتي الدوديسيل كبريتات صوديوم والايثيديوم بروميد في محاولة لتحييد محتواها البلازميدي.كانت النتيجة عند تحييد البلازميد باستخدام الايثيديوم بروميد عند تركيز ١,٢٥%. ٧ من العزلات اصبحت حساسة لعقار السيفترايكسون، بينما ال ٣ عزلات الاخري مازالت مقاومة لهذا العقار. عند استعمال الدوديسيل كبريتات صوديوم لتحييد البلازميد عند تركيز ١,٢% وجد ان عزلة واحدة فقط من البروتيس ميرابليس اصبحت حساسة لعقار السيفترايكسون ، بينما باقي العزلات لم يتم تحييد محتواها البلازميدي.