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Full Length Research Paper

Clinical microbiology study of diabetic foot ulcer in Iran; pathogens and antibacterial susceptibility

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The aim of this study was to investigate microbial pathogens and their antibiotic susceptibility profile in infected diabetic foot ulcers in Iranian patients. This was a one-year cross sectional study on diabetic patients with infected diabetic foot ulcer at Shariati Teaching Hospital, Tehran, Iran. Grade of ulcer was determined by Wagner's criteria. Specimens were obtained from the base of ulcer, deep part of the wound or aspiration and were tested with gram staining and antibacterial susceptibility was determined with both disk diffusion and E-Test methods. Total of 546 pathogens were isolated from 165 ulcers of 149 patients. Gram positive aerobes including *Enterococcal* species and methicillin resistant *Staphylococcus aureus* (*S. aureus*) (21.4 and 19.4%, respectively) were identified as the most common pathogens followed by Gram negative isolates including *Escherichia coli* and *Pseudomonas-aeruginosa* (12.6 and 5.4%, respectively). The majority of wounds were classified as Wagner grades 2 and 3 (15.7 and 75.7%). Appropriate empiric treatment to cover both these Gram positive and Gram negative pathogens is crucially important.

Key words: Diabetic foot ulcer, *Enterococcus*, *Staphylococcus*.

INTRODUCTION

Foot ulceration and infection in diabetic patients is one of the major causes of morbidity, hospitalization and foot amputation (Lipsky et al., 2004). This complication accounts for approximately 20% of hospital admissions in diabetic patients (Bild et al., 1989; Abdulrazak et al., 2005). Diabetic foot infection leads to approximately 50% of non traumatic lower limb amputations in the United States (Abdulrazak et al., 2005).

Diabetic foot infections include cellulitis, abscess, necrotizing fasciitis, septic arthritis, tendonitis and osteomyelitis. According to the previous studies, aerobic Gram positive cocci including *Staphylococcus aureus* and beta-hemolytic *Streptococci* are the major pathogens in the acute skin and soft tissue infections. *Enterococci*, Enterobacteriaceae and *Pseudomonas* are important

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pathogens in chronic ulcers (Bild et al., 1989; Gerding, 1995; Urbancic and Gubina, 2000; Abdulrazaket al., 2005). In a European study on 78 diabetic patients, *S. aureus* (42.3%), Enterobacteriaceae (12.5%), coagulase negative Staphylococcal species (10.6%) and *Pseudomonas aeruginosa* (10.6%) were the most frequent organism isolated from foot infections (Bild et al., 1989).

Shariati hospital is one of the major medical centers affiliated to Tehran University of Medical Sciences. One of the core divisions in this teaching hospital is Endocrinology and Metabolism Research Center. The diabetes clinic of this center takes care of a considerable number of diabetic patients including those with diabetic foot infections. Designing an appropriate protocol for empiric antibacterial treatment for diabetic foot infections involves a multidisciplinary team work including endocrinologists, infectious diseases specialists, medical microbiologists, clinical pharmacists and nurses. However, yet there is no comprehensive national protocol and guideline for empiric treatment of infected diabetic foot ulcer (considering microbial pathogens isolated in our patients) in Iran.

This issue urged to investigate microbial pathogens responsible for diabetic foot infection. So, the aim of this study was to determine microbial and antimicrobial susceptibility profile of infected diabetic foot ulcer in Iranian patients.

MATERIALS AND METHODS

The total number of 149 diabetic patients with infected foot ulcer participated in this cross sectional study at Endocrinology and Metabolism Research Center, Shariati hospital, Tehran, Iran, from January 2008 to January 2009. All patients were admitted to outpatient diabetes clinic inside the hospital campus.

Patients with clinical diagnosis of diabetic foot infection including superficial infected ulcers and osteomyelitis were included in the study. Patients who had received antibiotics (oral, topical, injection) within the previous week were excluded from the study.

After explanation of the study details and aims, written informed consent was obtained. The study protocol was approved by the ethics committee of Endocrinology and Metabolism Research Center in accordance with Helsinki declaration and the guidelines of Iranian Ministry of Health and Medical Education.

Wagner's criteria were used for ulcer grading. The wound size, depth and its infection status were graded (Oyibo et al., 2001; Armstrong and Peters, 2001; Weigelt et al., 2009) also.

Radiologic and imaging evaluation also were done by a simple foot X-ray and a triphasic bone scan (using 20 mCi Tc 99 m) of the whole body to rule out osteomyelitis. A venous blood sample was taken after overnight fasting for assessment of biochemical parameters.

Microbiology and susceptibility testing

After washing the wound with normal saline, the specimens were obtained from the base of the ulcer and deep part of the wound or by needle aspiration from the abscess. The specimens in thioglycollate tubes were sent to the microbiology laboratory and incubated at 37℃ for 24 h. After Gram staining, the cultures on blood agar and MacConkey agar were incubated under aerobic and anaerobic conditions at 37℃ for 48 h.

Antibiotic susceptibility tests were done by both disk diffusion and Epsilometer test (E-test) methods on incubated isolates onto Mueller-Hinton agar plates (transferred from broth achieving 0.5 McFarland visual turbidity standard) (Citron et al., 1991). E-test strips were obtained from AB BioMerieux Company, Solna, Sweden and the antibiotic disks were obtained from HiMedia Company, Mumbai. For this purpose, E-test strips and antibiotic disks were applied on separate plates and incubated for 24 h to evaluate the isolates' susceptibility to antibiotics. The following antibiotic disks were used to assess susceptibility: ciprofloxacin 5 mcg/disk, penicillin 10 mcg/disk, ceftriaxone 30 mcg/disk, cephalotin 30 mcg/disk, imipenem 10 mcg/disk, cefoxitin 30 mcg/disk, ceftizoxime 30 mcg/disk, ticarcillin- clavulanate 75 to 10 mcg/disk, metronidozole 5 mcg/disk, meropenem 10 mcg/disk, gentamicin 10 mcg/disk, amikacin 30 mcg/disk, clindamycin 2 mcg/disk, erythromycin 15 mcg/disk, ampicillin 10 mcg/disk, vancomycin 30 mcg/disk, ceftazidime 30 mcg/disk.

For E-test we used clindamycin 32 mcg, ceftazidime 256 mcg, vancomycin 256 mcg, meropenem 32 mcg strips. In the case bacterial occurred along the entire E-test strip, minimal inhibitory concentration (MIC) was reported as more than the highest value on the strip. If an E-test MIC value fell in-between two-fold dilutions, it was rounded up to the next upper value.

Methicillin resistant *Staphylococcus aureus* (MRSA) was defined as *S. aureus* isolates resistant to cefoxitin by using disk diffusion susceptibility test.

Statistical analyses

Quantitative variables were expressed as means ± SD and qualitative variables were expressed as percentage. The association between independent variables with MRSA and methicillin sensitive *Staphylococcus aureus* (MSSA) was tested by using student's t-test and Chi square or Fisher's exact test as appropriate. Multiple logistic regression model with enter method was fitted to explore independent predictors of MRSA infections. The odds ratios (ORs) with 95% confidence interval (CI) were calculated for MRSA associated ulcers. A P-value less than 0.05 was considered as statistically significant. Analyses were conducted by using STATA/SE 10.0 software.

RESULTS

Sixty percent of patients were male. Most of the patients were older than sixty years (45.7%). Eighty seven point nine percent of patients had type 2 diabetes. Duration of diabetes in 72.1% of the patients was equal or longer than 10 years and duration foot ulcers in 77.9% of patients was between one month and a year. Sixty two point nine percent of patient had poor diabetes control (HbA_{1C} of 8% or higher). The majority of wounds were classified as Wagner grades 2 and 3 (15.7 and 75.7%, respectively).

Total of 546 pathogens was isolated from 165 diabetic foot lesions of 149 patients with an average of 3.3 pathogens per lesion. Gram positive aerobic agents including *Enterococcus* species and *S. aureus* (21.4 and 19.4%, respectively) followed by Gram negative aerobic agents including *Escherichia coli* (*E. coli*) and

Table 1. Frequency of each isolated bacteria from diabetic foot wounds (number of patients = 149).

Bacterial category	Frequency (%)
n, isolates	546
Aerobic and facultative isolates	536(98.1)
Gram positive	338(61.9)
Streptococcus-spp.	17(3.1)
Group D *Strep-Entrococcus*	117(21.4)
Group D *Strep-Non Entrococcus*	14(2.5)
S. aureus	106(19.4)
Staphylococcus epidermidis	68(12.4)
Staphylococcus hemolyticus	14(2.5)
Micrococcus-spp	2(0.3)
Gram negative	198(36.2)
E. coli	69(12.6)
Citrobacter-spp	12(2.1)
Kebsiella-spp	16(2.9)
Pseudomonas-spp	4(0.7)
Pseudomonas-aeroginoza	30(5.4)
Klebsiella pneumoniae	15(2.7)
Acinetobacter-spp	28(5.1)
Citrobacter-freundai	6(1.01)
Entrobacter-spp	10(1.6)
Morganella-spp	7(1.1)
Proteus Mirabillis	1(0.9)
Anaerobic isolates	10(1.8)
Pepto Streptoccocus	9(1.6)
Bactereides Fragilis	1(0.1)

Pseudomonas-aeruginosa (12.6 and 5.4%, respectively) were the most common pathogens in this population. Polymicrobial infection was seen in 89.4% whereas single pathogen etiology was seen in 9.3% of all cases. One point eight percent of all bacterial isolates were identified as anaerobes. Table 1 shows the frequency of all isolated bacteria from foot ulcers.

Enterococcal species were the most common isolated bacteria from foot ulcers which majority of them (52.1%) were obtained from superficial wounds. Fifty three point one percent of MRSAs was isolated from superficial wounds versus 46.9% isolated from deep part of the wounds.

Based on susceptibility test results (E-Test) 91.4% of Enterococcal species were susceptible to vancomycin. 31.1% of *S. aureus* was MRSA which all of them were sensitive to vancomycin while 78.7% were resistant to clindamycin. About 94% of *streptococcal* isolates were susceptible to vancomycin.

All *P. aerogenosa* isolates were resistant to ceftazidime while 10% of those isolates were resistant to meropenem. 43.1% of *E. coli* and none of *Klebsiella* species were resistant to ceftazidime whereas both organisms were susceptible to meropenem. As predicted, the rate of susceptibility to meropenem was higher than third generation cephalosporins (ceftazidime).

Demographic characteristics and risk factors of participants and their relationship with frequency of MRSA and MSSA infections were shown in Table 2. Patients with duration of diabetes longer than 10 years had significantly higher risk for MRSA infections (OR = 1.28, 1.06 to 1.60). Also there was a significant relationship between hyperlipidemia and the frequency of MRSA infections (OR = 4.05, 1.17 to 14). In Wagner grades 2 and 3 wounds the most common isolated bacteria was *Enterococcus*.

The susceptibility of anaerobic bacteria were evaluated by both E-Test and disk diffusion methods. Tables 3 and 4 show the results obtained from each of these two methods for both aerobic and anaerobic isolates.

DISCUSSION

In this study, a comprehensive evaluation of microbiological profile and antimicrobial susceptibility of

Table 2. Relationship between risk factors with frequency of MRSA and MSSA infections.

Characteristic		MSSA n (%) N = 122	MRSA n (%) N = 16	P-value	OR	CI% 95
Sex	Female	41(33.6)	7(43.8)	-	1	-
	Male	81(65.9)	9(56.3)	0.45	0.66	0.23 - 1.91
Age(year)		59.23 ± 11.02	54.12 ± 9.50	0.08	0.95	0.91 - 1.00
<50		26(21.3)	6(37.5)	-	1	-
50-59		37(30.3)	5(31.3)	0.41	0.58	0.16 - 2.12
>=60		59(48.4)	5(31.3)	0.12	0.23	0.10 - 1.31
Type of diabetes	Type 1	16(13)	1(6.3)	-	1	-
	Type 2	106(86.8)	15(93.8)	0.45	2.24	0.27-18.15
Duration of diabetes		15.19 ± 8.81	16.50 ± 11.89	0.60	1.01	0.96-1.07
<10 years		89(74.2)	11(68.8)	-	1	-
≥10 years		31(25.8)	5(31.1)	0.02*	1.28	1.06-1.60
Duration of Ulcer (month)		5.44 ± 9.13	7.28 ± 8.52	0.46	1.01	0.97-1.06
< 1month		13(10.8)	1(6.3)	-	1	-
1-11 months		97(80.8)	11(68.8)	0.72	1.47	0.17 - 12.37
≥12 months		10(8.3)	4(25)	0.16	5.20	0.5 - 54.00
Complications	Hypertension	56(46.7)	8(50)	0.42	0.61	0.19 - 2.01
	Hyperlipidemia	48(40)	11(68.8)	0.02*	4.05	1.17 - 14.00
	Osteomyelitis	99(82.5)	14(87.5)	0.61	1.50	0.30 - 7.38
	Smoking	48(40)	11(68.8)	0.58	1.40	0.41 - 4.75
HbA_1C (%)		8.92 ± 1.92	8.52±1.75	0.42	0.88	0.66 - 1.19
<7%		20(16.8)	3(18.8)	-	1	-
7-7.9%		22(18.5)	8(18.8)	0.91	0.90	0.16 - 5.03
≥8%		77(64.7)	10(63.5)	0.83	0.86	0.31 - 3.44
FBS(mg/dl)		179.72 ± 74.27	180.43 ± 68.68	0.97	1.00	0.99 - 1.007
<126		30(25.2)	3(18.8)	-	1	-
126-175		31(26.1)	6(37.5)	0.38	1..93	0.44 - 8.45
176-226		35(29.4)	3(18.8)	0.85	0.85	0.16 - 4.56
>226		23(19.3)	4(25)	0.50	1.73	0.35 - 8.54
Depth of ulcer(cm)		0.47 ± 0.90	0.71 ± 0.89	0.31	1.28	0.80 - 2.08
≤0.5		77(64.7)	7(43.8)	-	1	-

Table 2. Cont.

>0.5	42(35.3)	9(56.3)	0.19	2.52	0.61 - 10.3
Size of ulcer(cm^2)	8.53 ± 24.51	7.15 ± 7.01	0.82	0.99	0.96 - 1.02
≤4	64(57.8)	7(43.8)	-	1	-
>4	55(46.2)	9(56.3)	0.45	1.49	0.52 - 4.28

MRSA = Methiciline resistant s. aureus; MSSA = Methiciline sensitive s. aureus. *, Significant.

Table 3. E-Test, susceptibility results of Gram positive, Gram negative and anaerobic isolates.

E-Test	Vancomycin n (%)	Clindamycin n (%)	Metronidazole
Gram positive isolates			
MRSA	33 (100)	5 (15.1)	
MSSA	75 (98.6)	50 (65.7)	
S. Epidermidis	60 (88.2)	31 (45.5)	
Group D Strep-*Entrococcus*	107 (91.4)		
Streptococcus-spp	16 (94.1)		
Group D Strep-Non *Entrococcus*	12 (85.7)		
S. aureus	106 (99.5)	55 (51.8)	
S. hemolyticus	14 (100)	10 (71.4)	
Micrococcus-spp	2 (100)	2 (100)	
Gram negative isolates	**Merpenem**	**Ceftazidim**	
Pseudomonas-aeroginoza	27 (90)	30 (100)	
E. coli	69 (100)	28 (40)	
Entrobacter-spp	10 (100)	8 (80)	
Acinetobacter-spp	16 (57.1)	16 (57.1)	
Citrobacter-spp	12 (100)	8(66.6)	
Kebsiella-spp	14 (87.5)	14(87.5)	
Pseudomonas-spp	4 (100)	0	
Klebsiella pneumonia	15 (100)	13 (86.6)	
Citrobacter-freundai	6 (100)	4 (66.6)	
Morganella-spp	6 (85.7)	7 (100)	
Proteus mirabilis	1 (100)	1 (100)	
Anaerobic isolates	**Clindamycin**	**Merpenem**	
Pepto Streptoccoccoc	5 (55.5)	9 (100)	0
Bactereides Fragilis	0	1 (100)	1 (100)

Table 4. Disk diffusion, susceptibility results (number and percentage) of Gram positive, Gram negative isolates and anaerobic isolates.

Characteristic	*Citrobacter* spp	*P. aeruginosa*	*Klebsiella pneumoniae*	*Klebsiella* spp	*Enterobacter* spp	*E. coli*	*Acinetobacter* spp
Gram negative							
Ciprofloxacin	10 (83.3)	27 (90)	9 (60)	14 (87.5)	8 (80)	17(24.6)	18 (64.2)
Ceftazidim	10(83.3)	29(96.6)	4(26.6)	14(87.5)	9(90)	24(34.7)	11(39.2)
Merpenem	12(100)	29(96.6)	15(100)	14(87.5)	10(100)	66(95.6)	25(89.2)
Imipenem	12(100)	30(100)	15(100)	14(87.5)	10(100)	69(100)	19(67.8)
Amoxi/clave	2(18.1)	0	0	1(6.2)	0	3(4.3)	2(7.1)
Ceftriaxone	10(83.3)	0	12(80)	10(62.5)	10(100)	21(30.4)	6(21.4)
Ceftizoxim	10(83.3)	1(3.3)	14(93.3)	14(87.5)	10(100)	38(55)	14(50)
Gram positive	***S. aureus***	***S. epidermidis***	***Streptococcus* spp**	***Enterococcus* spp**			
Vancomycin	106(100)	68(100)	17(100))	62(64.9)			
Cefoxitin	73(68.8)	48(70.5)					
Ciprofloxacin	59(55.6)	37(54.4)					
Merpenem	100(94.3)	60(88.2)					
Clindamycin	35 (33)	27 (35.2)	11(64.7)	0			
Amoxi/clave	28 (26.4)	33 (48.5)					
Ampicillin			15(88.2)	113(96.5)			
Ceftraxone			8(47)	5(4.2)			
Amikacin			1(5.8)	4(3.4)			
Ciprofloxacin			8(48)	54(46.1)			
Penicillin			14(82.3)	32(27.3)			
Gentamicin			6(35.2)	50(42.7)			
Anaerobic isolates	***Pepto Streptococci***	***Bactereides Fragilis***					
Vannomycin	9(100)	0					
Cefalotin	9(100)	1(100)					
Ceftizoxim	9(100)	0					
Merpenem	9(100)	0					
Clindamycin	5(55.5)	0					
Imipenem	9(100)	0					
Ampicillin	9(100)	0					
Ceftriaxone	4(44.4)	0					
Amikacin	2(22.2)	1(100)					
Cotimoxazole	7(77.7)	0					
Penicillin	9(100)	0					
Gentamicin	0	1(100)					
Erythromaycin	7(77.7)	0					
Metronidazole	2(22.2)	0					

infected diabetic foot ulcer in diabetic patients referred to diabetic clinic was done. Univariate analysis showed significant association of hyperlipidemia, duration of diabetes and age (years) with prevalence of MRSA whereas duration of diabetes and hyperlipidemia were the only independent risk factors of MRSA infections in multivariate analysis. This is while according to the results, duration of diabetic ulcer, type of diabetes and HbA_1C level did not have significant effect on the prevalence of MRSA infections.

In an Indian study on 80 patients with Wagner's grades 3 to 5 diabetic foot ulcers, aerobic Gram negative organisms (51.4%) and aerobic Gram positive organisms (33.3%) were the most common isolated pathogens. In that study, over 70% of the patients were positive for multidrug resistant organisms including extended spectrum beta-lactamase (ESBL) positive bacteria and MRSA (Gadepalli et al., 2006).

In a recent study on 440 diabetic patients with diabetic foot infection in Kuwait, 777 pathogens were isolated. In that study, the most common pathogens isolated from the lesions were aerobic Gram-negative bacteria (51.2%), Gram-positives (32.3%) and anaerobes (15.3%), respectively.

The finding of this study is something different from ours. In our study in contrast to Kuwait study, Gram positive bacteria were the most common isolated pathogens whereas in that study Gram negatives were the most common isolates. The most common Gram negative organism in Kuwait study was *P. aeruginosa* while it was the second common organism in our study. In that study, *S. aureus* was detected as the most common Gram positive bacteria whereas in our study it was the second most common pathogen and *E. coli* was the first. Polymicrobial infection in that study (75%) was less than our study (89.4%). This comparison shows that although both countries (Iran and Kuwait) are in the same region but the microbial pattern of diabetic foot infection is different.

In a study conducted by Raja (2007) in Malaysia on 194 patients, 287 pathogens were isolated that like Kuwait study Gram Negative bacteria *(Proteus* species and *P. aeruginosa*) were predominant which is different from our findings. The most frequent detected organisms in that study are different from ours (Raja, 2007).

Average number of pathogens per lesion in our study (3.3) was more than Kuwait, Malaysia and even United State studies (1.8, 1.47 and 2.7%, respectively) (Citron et al., 2007; Raja, 2007; Benwan et al., 2012).

An Iranian prospective study on 32 diabetic patients in 2006, revealed polymicrobial etiology in 50% of the patients. Aerobic Gram negative rods (54.8%) and gram positive *cocci* (42.9%) were frequent isolates. All cultured microorganisms showed high resistance to the antibiotic treatments used in the study. The highest resistance against antibacterial agents was seen in *S. aureus* and *P. aeruginosa* (Alavi et al., 2007).

A number of other previous studies concluded that Gram positive aerobes were responsible for the majority of diabetic foot ulcer infections in their patients (Mantey et al., 2000; Fejfarova et al., 2002). Although in the present study *Enterococcal* species were the most common pathogens isolated from diabetic foot ulcer, other studies like Joseph et al. study, showed group B *Streptococci* as the predominant cause of diabetic foot infections (Joseph, 1991). In this study, as it was predicted, the Gram negative isolates including *Pseudomonas* and *Klebsiella* species showed higher resistance to third generation cephalosporins than carbapenems and this could be due to the large number of prescriptions of cephalosporins over a long period of the time for diabetic patients with foot ulcers. Antimicrobial susceptibility in our study was somewhat similar to Raja (2007) study.

The difference in findings in different studies (Citron et al., 2007; Raja, 2007; Benwan et al., 2012) may be due to the difference in sample size, specimen collection method, site of specimen collection, microbial detection method, antimicrobial agent used and geographical region and culture.

Conclusion

In conclusion, according to the results, *Enterococci* and *Staphylococci* were the most common pathogens in the infected diabetic foot ulcers followed by Gram negative aerobes like *E. coli* and *Klebsiella* species. The difference in microbial pattern of diabetic foot infection in various studies shows that the empirical therapy in each country should be selected considering the most common specific pathogen of the region and its antimicrobial susceptibility. Since this study was performed on outpatients and based on the susceptibility results, it seems it is crucially important to start such an empiric antibacterial treatment to cover both Gram positive (including MRSA) and Gram negative bacteria. Antimicrobial susceptibility results showed that vancomycin and merpenem may be appropriate agents for empirical therapy in Iran.

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