Original Article

Increasing incidence of Gram-negative organisms in bacterial agents isolated from diabetic foot ulcers

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Abstract

Introduction: In the present study, we sought to identify the bacterial organisms associated with diabetic foot infections (DFIs) and their antibiotic sensitivity profiles.

Methodology: We retrospectively reviewed the records of wound cultures collected from diabetic patients with foot infections between May 2005 and July 2010.

Results: We identified a total of 298 culture specimens (165 [55%] wound swab, 108 [36%] tissue samples, and 25 [9%] bone samples) from 107 patients (74 [69%] males and 33 [31%] females, mean age 62 ± 13 yr) with a DFI. Among all cultures 83.5% (223/267) were monomicrobial and 16.4% (44/267) were polymicrobial. Gram-negative bacterial isolates (n = 191; 61.3%) significantly outnumbered Gram-positive isolates (n = 121; 38.7%). The most frequently isolated bacteria were *Pseudomonas* species (29.8%), *Staphylococcus aureus* (16.7%), *Enterococcus* species (11.5%), *Escherichia coli* (7.1%), and *Enterobacter* species (7.1%), respectively. While 13.2% of the Gram-negative isolates were inducible beta-lactamase positive, 44.2% of *Staphylococcus aureus* isolates were methicillin resistant.

Conclusions: Our results support the recent view that Gram-negative organisms, depending on the geographical location, may predominate in DFIs.

Key words: diabetic foot infection; bacterial pathogens; culture; Gram-positive bacteria; Gram-negative bacteria

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Introduction

Diabetes, with its increasing prevalence and incidence, is regarded as a global health problem. Today, it affects approximately 171 million people worldwide and this number is estimated to reach 366 million in 2030 [1]. In Turkey, 7.2 % of the population has diabetes [2]. Diabetic foot infections (DFIs) are associated with significant mortality and morbidity and are the leading cause of non-traumatic lower extremity amputations [3-5].

Because the results of wound cultures are available in one to three days on average, comprehensive empirical antimicrobial therapy covering the most probable causative agents is a key factor in the management of DFIs. Moreover, given the fact that many patients receive antimicrobial treatment prior to wound sampling, some cultures may yield false negative results and clinicians may have to rely solely on their clinical consideration.

Microbiological studies of DFIs conducted so far have yielded inconsistent results. This discrepancy might be attributed to the varying methodological design and quality among studies. The prevailing belief that *Staphylococcus aureus* is the predominant pathogen in DFIs has been derived mainly from studies undertaken in Western countries [5,6]. Recent studies from Eastern countries, however, have raised skepticism about this assumption [7-10]. Turkey is a transition point between the Eastern and Western countries. In the current study, we sought to demonstrate the microbiological profile and antibiotic susceptibility patterns of organisms isolated from diabetic patients in a tertiary hospital in Turkey.

Table 1. The distribution of bacteria isolated from diabetic foot infections

Bacteria	N	%[a]	%[b]
Gram-positive bacteria			
Staphylococcus aureus (MS)[c]	29	9.3	24.2
Staphylococcus aureus (MR)[c]	23	7.4	19.2
Enterococcus spp	36	11.5	30.0
Staphylococcus (coagulase negative)	16	5.1	13.3
Micrococcus spp	9	2.9	6.7
Streptococcus spp	8	2.6	6.7
Total	121	38.7	100
Gram negative bacteria			
Pseudomonas spp	93	29.8	48.7
Enterobacter spp	22	7.1	11.5
Escherichia coli	22	7.1	11.5
Klebsiella spp	12	3.8	6.3
Proteus spp	15	4.8	7.9
Acinetobacter spp	8	2.6	4.2
Other Gram negatives[d]	19	6.1	9.9
Total	191	61.3	100

[a] Rate within all isolates

[b] Rate depending on Gram staining

[c] MS, methicillin-sensitive; MR, Methicillin-resistant

[d] *Citrobacter* spp., *Serratia* spp, *Stenotrophomonas* spp, *Burkholderia* spp, *Morganella morganii*, *Pantoea agglomerans*, *Edwardsiella tarda*, *Providencia rustigianii* (0-1.4%)

Methodology

The records of diabetic patients with foot infections admitted to the Underwater and Hyperbaric Medicine Center between May 2005 and July 2010 were reviewed and those with a wound culture, assessed by the Infectious Diseases and Clinical Microbiology (IDCM) Laboratory, were enrolled in the study. Wound cultures from both inpatients and outpatients were included. Both centers are affiliated with Gulhane Military Medical Academy Haydarpasa Teaching Hospital, which is a referral center for diabetic patients with foot infections in Istanbul, Turkey.

We obtained a wound culture from a diabetic patient if he/she had clinical signs of wound infection on the day of admittance; cultures were repeated when clinically indicated. Wound culture results were presented in Table 1. The Ethical Committee of Gulhane Military Medical Academy Haydarpasa Teaching Hospital approved the study protocol (#2012-26).

Swab samples were obtained using sterile swabs, following the removal of debris-containing tissues and cleansing the wound and peri-wound with sterile normal saline. Deep tissue samples were obtained from the viable and non-viable tissue junction using a curette or punch biopsy material. We obtained bone specimens during surgical debridement using a rongeur whenever possible.

The IDCM laboratory performed microorganism identification and antibiotic sensitivity testing. The specimens were incubated at 37^{o}C for 24 to 48 hours on eosin methylene blue, chocolate and 5% sheep blood agars. Microorganisms were identified by standard methods based on the morphology of the colonies, microscopic appearance of bacteria, Gram-staining, and by using rapid Gram-positive and -negative identification kits (BBL Crystal Identification System, Becton Dickinson Co, Cockeysville, Md:, USA). Anaerobic culturing was not routinely performed in the IDCM laboratory.

Antibiotic sensitivity testing was performed by the Kirby-Bauer disc diffusion method and interpreted in accordance with Clinical and Laboratory Standards Institute (CLSI, 2005) guidelines [11]. The procedure involved swabbing of 0.5 McFarland testing

Table 2. Percentage of in vitro susceptibility of Gram-positive aerobic organisms to antimicrobials (%)[a]

Bacteria	CIP	SAM	SXT	VA	MET	LNZ	FA	TE	P	E	LEV
Staphylococcus aureus (MS)	71	100	50	100	100	100	100	72	48	71	95
Staphylococcus aureus (MR)	35	50	56	100	0	90	100	55	13	34	0
Enterococcus spp.	30	90	26	97	9	93	61	11	79	19	60
Staphylococcus (coagulase negative)	28	50	61	100	12	93	100	28	20	25	57
Micrococcus spp.	33	20	60	100	50	100	80	50	37	25	0
Streptococcus spp.	50	40	40	100	16	83	60	0	25	50	100

[a] CIP, ciprofloxacin; SAM, Ampicillin/sulbactam; SXT, trimethoprim-sulfamethoxazole; VA, vancomycin; MET, methicillin, LNZ, linezolid, FA, fusidic acid, TE, tetracycline, P, penicillin; E, erythromycin, LEV, levofloxacin.

microorganism over ceftazidime (30 µg) and ceftazidime/clavulanic acid (30/10 µg) discs, placed on a Mueller Hinton agar plate. Any microorganism (*Klebsiella pneumoniae*, *K. oxytoca, Escherichia coli* and *Proteus mirabilis)* was considered as extended spectrum beta-lactamases (ESBL) positive if there was an increase ≥ 5 mm in zone diameter with ceftazidime/clavulanate versus its zone size when tested with only ceftazidime disc. Inducible beta-lactamases (IBL) positive microorganisms were identified using the double disc method according to Sanders and Sanders [12]. Cefoxitin was used as inducer of these beta-lactamases. The induction of beta-lactamase was indicated by the occurrence of antagonism between the cefoxitin disc and the antibiotic being tested. Methicillin resistance was tested by using the cefoxitin disc diffusion method (30 µg). An inhibition zone diameter of ≤ 21 mm for *S. aureus* and ≤ 24 mm for coagulase-negative *Staphylococcus* was reported as methicillin resistant.

Results

A total of 298 wound cultures from 107 patients with diabetic foot ulcers were identified. There were 74 (69%) male and 33 (31%) female patients. The mean age of the patients was 62 ± 13 years (range: 18 to 95 years), and the mean HbA1c was 9 ± 2.5 % (range: 4.8% to 16.4 %).

Of the 298 samples, 165 (55%) were wound swabs, 108 (36%) were deep tissue samples, and 25 (9%) were bone specimens. Thirty-one samples did not show any bacterial growth. Among 267 culture-positive samples, a total of 312 aerobic bacteria were identified. Polymicrobial isolates were detected in 44 (16%) samples. The average number of isolates per culture-positive sample was 1.16.

The distribution of isolated bacteria is shown in Table 1. There were 121 (38.7%) Gram-positive isolates and 191 (61.3%) Gram-negative isolates. Among all isolates, *Pseudomonas spp.* was the most frequent bacteria (n = 93; 29.8%), followed by *S.aureus* (n = 52; 16.7%) and *Enterococcus spp* (n = 36; 11.5%).

Among Gram-positive isolates, *S. aureus* was the most frequently isolated species (43.4%). Of these, 44.2% were methicillin-resistant *S. aureus* (MRSA). The second most frequent Gram-positive organism was *Enterococcus spp.* (n = 36; 30%). Coagulase-negative *Staphylococci*, which are usually recognized as colonizers, were isolated in 16 (13.3.%) wound cultures.

Pseudomonas species were the most frequently isolated bacteria among Gram-negatives (48.7%) followed by *Enterobacter* spp (11.5%) and *Escherichia coli* (11.5%). While 30 (32.2%) of the *P. aeruginosa* and 15 (17.6%) of the *Enterobactericeae* species demonstrated IBL activity, two *Escherichia coli* and one *Klebsiella oxytoca* species expressed ESBL activity.

In-vitro sensitivities of isolated bacteria are illustrated in Tables 2 and 3. While one of the *E. faecalis* species demonstrated vancomycin resistance (VREF), the remaining Gram-positive species were sensitive to vancomycin. Fusidic acid was efficient against all *Staphylococcus* species including those with methicillin resistance. Most of the Gram-negative isolates were sensitive against sulbactam/cefoperazone and tazobactam/piperacillin. Most isolates were also sensitive against ceftadizime, amikacin and imipenem. Two (25%) of the *Acinetobacter* spp isolates had imipenem resistance

Table 3. Percentage of in vitro susceptibility of Gram-negative aerobic organisms to antimicrobials (%)[a]

Bacteria	AK	CIP	CTX	CAZ	AMC	SXT	TPZ	IPM	CES	ATM
Pseudomonas spp.	68	40	12	63	5	19	93	50	73	56
Enterobacter spp.	86	77	71	76	9	68	100	76	100	62
Escherichia coli	85	45	59	81	31	33	100	100	100	78
Klebsiella spp.	81	27	58	75	41	27	100	83	100	63
Proteus spp.	92	86	93	93	66	66	100	100	100	91
Acinetobacter spp.	57	16	0	37	0	25	12	71	50	75
Other Gram-negatives[b]	64	40	64	88	47	37	75	94	100	64

[a]AK, amikacin, CIP, ciprofloxacin; CTX, cefotaxime, CAZ, ceftazidime; AMC, amoxicillin / clavulanic acid, SXT, trimethoprim / sulphamethoksazol; TPZ, tazobactam / piperacillin, IPM, imipenem, CES, sulbactam / cefoperazone, ATM, aztreonam

[b] *Citrobacter* spp., *Serratia* spp, *Stenotrophomonas* spp, *Burkholderia* spp, *Morganella morganii*, *Pantoea agglomerans*, *Edwardsiella tarda*, *Providencia rustigianii* (0-1.4%).

Discussion

Since the early 1980s, DFIs are recognized to be polymicrobial in nature. Gram-positive cocci are almost always the most commonly isolated organisms, followed by Gram-negative and anaerobic bacteria. The majority of the studies conducted during the last two decades in Western countries have shown that unless antibiotics have been used prior, cultures from acute diabetic foot wounds grow a single pathogen, which is usually *S. aureus* or *Streptococcus* spp [13].

In the current study, 312 bacteria were isolated from 267 specimens, with a rate of 1.16 isolates per culture (IPC). While these results compare favorably with several previous studies such as those by Hayat *et al*. (1.24 IPC) [14] and Viswanathan *et al*. (1.21 IPC) [15], they differ from several others, such as the investigation by Citron *et al*. [6], which revealed 2.7 IPC among aerobic and 2.3 IPC among anaerobic bacteria in a diabetic population involving 433 patients with foot infections.

Our finding that 61.3% of the overall isolates were Gram-negative aerobic agents is of note. Several studies from the recent literature have reported similar observations. Gadapelli *et al*. [7], Shankar *et al*. [8], Ramakant *et al*. [9] and Raja [10], from Eastern developing countries and Şerefhanoğlu *et al*. [16] and Örmen *et al*. [17] from Turkey, reported an increase in the prevalence of aerobic Gram-negative bacteria isolated from DFIs.

Another interesting and important observation of this study was the apparent predominance of *Pseudomonas* species, particularly among Gram-negative isolates (48.7%) but also among all isolates (29, 8%). This observation may be attributed in part to the chronic and/or recurrent characteristics of wound infections in our patient population. Several studies with large patient cohorts, especially those from Pakistan and India, have reported similar rates of *Pseudomonas* in diabetic patients with foot infections [7,8]. While Abdulrazak *et al*. [18] reported a 17.5% rate of *P. aeruginosa* among all isolates, Ramakant *et al*. [9] and Hayat *et al*. [14] reported rates of 27.05% and 20.1%, respectively. The majority of these studies have also reported a proportional increase in the prevalence of multi-drug resistance among pathogens identified from DFIs during the same period. In the current study, carbapenem sensitivity was 49.4% and multidrug resistant strains represented almost one third of all *Pseudomonas* species. Shankar *et al*. [8] from South India and Kandemir *et al.* [19] from Turkey reported 44% and 45% rates for multidrug resistant *P. aeruginosa* strains isolated from patients with DFIs, respectively. Kandemir *et al*. [19] demonstrated that risk factors such as the duration of past antibiotic use, prolonged hospitalization, and the presence of neuro-ischemic diabetic foot ulcers and osteomyelitis were closely associated with multiple drug resistance.

In a multi-center study ("SIDESTEP") conducted by Lipsky *et al*. [20], ertapenem, which is known to be ineffective against *Pseudomonas* species, was compared with piperacilin/tazobactam in diabetic patients with foot infections. Although some wound cultures involved *P. aeruginosa,* piperacilin/tazobactam and ertapenem protocols surprisingly revealed similar outcomes. The authors from this study, as well as several others from western countries, have pointed out that *P. aeruginosa* was a commensal organism rather than a causative pathogen and hence would not require any specific antimicrobial coverage. According to Lipsky *et al*., wound care measures such as avoiding moisture in the peri-wound environment, frequent changing of wound dressings,

and avoiding hydrotherapy-based wound care modalities would be sufficient to eradicate *P. aeruginosa*.

S. aureus has long been recognized as the predominant pathogen in DFIs. In the current study, however, it was the second most frequently isolated agent, coming after *P. aeruginosa.* Following the 1990s, community-acquired MRSA emerged as an important pathogen in DFIs, comprising between 12% to 40% of all *Staphylococcus* species [16]. We found a high rate (44.2%) of methicillin resistance in our series. Nevertheless, in a recent review, Lima *et al*. [21] reported an increase in antibiotic resistance among diabetic patients with foot infections and recommended the avoidance of wide empirical antibiotic coverage unnecessarily. Another important finding of this study was the fact that fusidic acid was efficient against all *S. aureus* species, including MRSA. Fusidic acid may be considered an important therapeutic alternative, particularly in mild to moderate DFIs.

In a recent study performed on diabetic patients with foot infections [22], while Gram-negative agents represented 38% of all organisms isolated from superficial wound cultures, they comprised almost twice the rate (67%) in patients with diabetic foot osteomyelitis. This finding suggests that the more chronic or complicated (*e.g.*, presence of osteomyelitis) DFIs are, the more Gram-negative agents will predominate [23].

Our study has several limitations. Because our hospital is a referral center for patients with DFIs, the majority of our patients had received several courses of antimicrobial treatment previously. However, due to missing data, we were not able to compare the relationship between antibiotic usage and culture results. Additionally, the current study is limited by the lack of anaerobic sampling. As humans live longer, the steady increase in diabetes and its associated complications such as DFIs will translate into a rising health-care burden worldwide. The traditional recognition that "DFIs are mostly caused by *S. aureus* or Gram-positive species" may not reflect a universal clinical feature, and geographic variances emphasize the need for local treatment guidelines [24]. This necessity has lately been demonstrated by many studies, including the current one, and those from Eastern countries, which reported a significant shift toward more Gram-negative organisms isolated from DFIs. Comprehensive empirical antimicrobial coverage is a key factor for successful DFI management; hence we need more, larger, prospective and controlled studies from all over the world to better address this issue.

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