Original Article

Anaerobic Culture of Diabetic Foot Infections: Organisms and Antimicrobial Susceptibilities

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Abstract

Introduction: The prevalence of diabetes mellitus is high in Singapore. Infections of the lower limb are significant causes of morbidity in this population. Although the aerobic bacteriology of these infections is well-documented, there is less data available on the anaerobic pathogens involved. This study sets out to describe the anaerobic bacteria associated with diabetic foot infections, and evaluates the susceptibility to 3 antimicrobials with anaerobic activity. Materials and Methods: Anaerobic culture was performed on operative samples taken from diabetic foot infections. Organisms were identified through standard microbiological methods and commercial identification kits. Antimicrobial susceptibility testing to clindamycin, metronidazole and imipenem was performed by agar dilution. Results: One hundred and two strains of strict anaerobic bacteria were isolated from 30 unique specimens. The predominant anaerobic isolates were *Peptostreptococcus* spp. (46%) and *Bacteroides fragilis* group (19%). Antibiotic resistance was detected for clindamycin (18%), metronidazole (1%) and imipenem (2%). Conclusion: Multiple anaerobic species can be isolated from diabetic foot infections. A significant proportion of isolates are resistant to clindamycin, while resistance to imipenem and metronidazole remains low.

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Introduction

Singapore has a relatively high prevalance of diabetes mellitus, with a recent survey showing an age-standardised prevalence of 7.8% in 2004.[1] Diabetic soft-tissue infections result in significant morbidity in this population of patients. The spectrum of disease ranges from infected foot ulcers, cellulitis to chronic osteomyelitis. Infections in diabetes are often polymicrobial, involving a mixture of aerobic and anaerobic flora.[2] Antibiotic therapy is often empirical and an antibiotic with anaerobic cover is often recommended.[3]

Antibiotic resistance in aerobic bacteria is of global concern; however, antibiotic resistance in anaerobes is often overlooked. With reports of resistance to anaerobic antimicrobials,[4,5] and variable antimicrobial resistance amongst anaerobic genera,[6] continued surveillance of anaerobic susceptibility patterns is vital to determine current and future trends.[7]

The aims of this study were to describe the anaerobic bacteriological flora of diabetic foot infections in Singapore, and to determine the antibiotic susceptibilities of these anaerobic isolates to 3 antibiotics with anaerobic activity.

Materials and Methods

Sample Collection

Only intraoperative samples collected from surgical procedures were included in the study, beginning from June 2006. Swab samples were transported to the laboratory using CultureSwab with Amies (BD, USA). Tissue and bone samples were transported to the laboratory in thioglycolate broth (BioMedia Laboratories, Malaysia). Culture samples were eligible for inclusion in the study if the patient was identified as a known diabetic patient, if the sample originated from the foot or lower limb, and if the clinical details on the request form indicated clinical suspicion of infection. If both tissue and swabs were received for a patient, only the tissue sample was included in the study results. Clinical details were also retrieved from the patient electronic database for every patient sample

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included in the study. Based on this data, infections were divided into 3 broad categories for the purposes of data analysis: soft tissue infections, soft tissue infections with gangrene, and osteomyelitis with overlying soft tissue infection.

Culture and Identification Methods

All samples were transferred into cooked meat media (BioMedia Laboratories, Malaysia) on receipt in the laboratory. Cooked meat media were incubated for 40 to 48 hours at 35°C in an ambient atmosphere. Following incubation, approximately 0.3 mL of the enrichment broth was aspirated and plated onto pre-reduced CDC anaerobic agar with 5% sheep blood (C plate), CDC anaerobic agar with 5% sheep blood and PEA, and CDC anaerobic agar with laked sheep blood, kanamycin and vancomcyin (all media from BD, USA) for anaerobic culture. All plates were incubated at 35 to 37°C in an anaerobic incubator (ThermoForma, USA). A separate C plate was simultaneously inoculated, and incubated in 5% CO_2 for 40 to 48 hours for growth comparison. Culture plates were removed after 40 to 48 hours incubation for inspection of growth, and re-incubated for a total of 6 days. Suspected growth of anaerobes was confirmed by aero-tolerance testing. Bacterial identification was performed using published algorithms[8, 9] and API 20 A (bioMérieux, France). Amplification of 16S ribosomal DNA was performed for isolates which we were unable to identify by conventional methods, using previously published primers.[10] The amplified region from base positions 54 to 510 for 16S rDNA were matched against entries in the PubMed BLAST database.

Susceptibility Testing

Only unique isolates from specimens collected during a single patient-episode were selected for susceptibility testing. Minimum inhibitory concentrations (MIC) to metronidazole, clindamycin and imipenem were obtained by the agar dilution method, performed according to CLSI methods.[11] Antibiotic powders, obtained either from the manufacturer or Sigma-Aldrich (Singapore), were dissolved in sterile ultra-pure water and added to supplemented brucella agar (Sigma-Aldrich, Singapore) with 5% defibrinated sheep blood (BioMedia Laboratories, Malaysia) to provide increasing 2-fold concentrations of antibiotic.

Bacterial suspensions in brucella broth (BD, USA) were prepared from fresh overnight cultures (or 40 to 48 hours cultures for slow-growing anaerobic isolates) and adjusted to a turbidity density of 0.5 MacFarland using a nephelometer (Biomerieux, France). The bacterial suspensions were applied to agar plates using a multipoint inoculator (Mast Diagnostics, England) to yield a final inoculum of 10^4 colony-forming units per spot. Agar dilution plates were incubated in anaerobic jars using anaerobic gas paks (BD, USA) and maintained at 35°C for 42 hours to 48 hours. MIC endpoints were read and susceptibility interpretations were applied using CLSI guidelines. American Type Culture Collection strains of *Bacteroides fragilis* ATCC® 25285 and *Eubacterium lentum* ATCC® 43055 were included as quality controls, and test values obtained were in line with published standards.[11]

Statistical Analysis

Data were analysed using Excel (Microsoft, Redmond, USA) while relative risk ratios and 2 x 2 chi-square analysis was performed by StatCalc (CDC, Atlanta). A *P* value of <0.05 was considered to be statistically significant.

Results

Anaerobes were cultured from 30 (79%) out of 38 samples received, which comprised tissue (n = 25), swabs (n = 11) and bone (n = 2). On average, 4 different anaerobic species were cultured from each sample. The most common anaerobic isolates were *Peptostreptococcus* spp. (47%) and *Bacteroides fragilis* group (19%) (Table 1). Three isolates were identified by 16S rDNA sequencing as *Eggerthella lenta* (98% similarity), *Fusobacterium gonidoformans* (98% similarity) and *Acidominococcus intestinalis* (98% similarity). Four anaerobic gram-positive bacilli and 1 gram-positive coccus remained unidentified following 16S rDNA sequencing. *Bacteroides* spp. were significantly more likely to be isolated from gangrenous soft-tissue infections than from other types of infections (relative risk 5.3, *P* <0.05). There were no significant differences for the other types of anaerobes between the 3 categories of infection.

One hundred and two unique isolates of anaerobes were available for susceptibility testing. Antibiotic susceptibilities for imipenem and clindamycin were available for 99 anaerobic strains, while susceptibilities for metronidazole were available for 97 strains. The remaining strains for which results are not available became non-viable during the course of testing. Eighty (81%) anaerobic isolates were susceptible to clindamycin, 97 (98%) isolates were susceptible to imipenem and 98 (99%) isolates were susceptible to metronidazole (Table 2). Clindamycin resistance was predominantly present in the *Bacteroides fragilis* group and peptostreptococci, while imipenem resistance was present in 2 *Fusobacterium* spp. Metronidazole resistance was only present in 1 *Peptostreptoccus* spp. isolate.

Discussion

To our knowledge, this is the first study to investigate the susceptibility of anaerobic isolates from clinical infections

Table 1. Anaerobes Isolated From Diabetic Foot Infections

Genus	Category of infection			Total (all types of infections)
	Gangrenous soft tissue infection	Osteomyelitis with overlying soft tissue infection	Soft tissue infection	
Peptostreptococcus spp.	14 (50%)	12 (35%)	22 (55%)	48 (47%)
Bacteroides spp.	11 (39%)	4 (12%)	4 (10%)	19 (19%)
Prevotella spp.	2 (7%)	7 (21%)	8 (20%)	17 (17%)
Veillonella spp.		2 (6%)	2 (5%)	4 (4%)
Gram-positive bacillus		3 (9%)	1 (3%)	4 (4%)
Porphyromonas spp.		1 (3%)	2 (5%)	3 (3%)
Clostridium spp.		2 (6%)		2 (2%)
Fusobacterium spp.		1 (3%)	1 (3%)	2 (2%)
Eggerthella spp.		1 (3%)		1 (1%)
Acidominococcus spp.		1 (3%)		1 (1%)
Gram-positive coccus	1 (4%)			1 (1%)
Total	28 (100%)	34 (100%)	40 (100%)	102 (100%)

* (% of total isolates in each category of infection)

Table 2. Antibiotic Susceptibilities of Anaerobic Isolates

	Clindamycin						Imipenem					Metronidazole				
	n*	MIC range	MIC90	%S	%I	%R	n	MIC range	MIC90	%S	%R	n	MIC range	MIC90	%S	%R
All tested anaerobes	99	≤0.03 - >16	>16	81	1	18	99	<0.06 - >32	0.5	98	2	97	<0.125 - >64	4	99	1
by organism group																
Bacteroides fragilis group	19	0.5 - >16		47	–	53	19	≤0.06 - 4		100	0	19	0.25 - 2		100	0
Clostridium spp.	2	0.25 - 1		100	–	0	2	0.25 - 4		100	0	2	≤0.125 - 0.25		100	0
Fusobacterium spp.	2	0.06 - >16		50	–	50	2	>32		0	100	2	0.25		100	0
Peptostreptococcus spp.	46	≤0.03 - >16		85	2	13	46	≤0.06 - 2		100	0	45	≤0.125 - >64		98	2
Porphyromonas spp.	3	≤0.03 - 0.25		100	–	0	3	≤0.06 - 0.125		100	0	2	≤0.125 - 4		100	0
Prevotella spp.	16	≤0.03 - >16		94	–	6	16	≤0.06		100	0	16	0.25 - 4		100	0
Veillonella spp.	4	≤0.03 - 0.125		100	–	0	4	≤0.06		100	0	4	≤0.125 - 8		100	0

MIC: minimum inhibitory concentration
* number tested

in Singapore. While resistance to metronidazole and imipenem remains low, 19% of isolates showed frank resistance or reduced susceptibility to clindamycin.

The anaerobes isolated from these diabetic foot infections are in line with other reported studies,[2,12] where peptostreptococci formed the predominant isolates. Although the exact role of anaerobic bacteria in these polymicrobial infections is still debated, expert opinion suggests that anaerobes are more likely to be isolated from long-standing or severe infections.[2] The impact of sampling

methods and transport media are also important, as multiple studies have demonstrated poor correlation for culture results from superficial wound swabs compared with deep tissue or bone samples.[13]

There is scant regional data for antimicrobial resistance in anaerobic isolates. This lack of data may be attributed to the relative difficulty in ensuring adequate sampling, sample transportation and culture conditions for this fastidious group of organisms, as well as the complexity of recommended susceptibility testing methods. Data from a 1992 Australian study showed that metronidazole, imipenem and ampicillin/sulbactam were the most active agents against anaerobic isolates,[14] and similar results were reported from Belgium[15] and the United States.[16] The latter 2 studies also reported increasing resistance rates for clindamycin and cefoxitin.

It is not known whether the susceptibilities for the study (which were isolated from diabetic soft-tissue infections) are representative of all anaerobic infections in Singapore. A multi-centre study from the United States reported that resistance rates were higher in blood-stream anaerobic isolates.[4]

The results of this study demonstrate that when appropriate specimens and transport media are used for sample collection from diabetic foot infections, multiple anaerobic species can be isolated. Metronidazole and imipenem resistance remains low in these isolates, but there is significant resistance to clindamycin. Further work will be required to clearly delineate antimicrobial resistance in clinically significant anaerobes isolated from other infections.

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