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Microbial content recovered from diabetic foot infections: a cross-sectional study in Brazil

Conteúdo microbiano recuperado em infecções de pé diabético: um estudo transversal no Brasil

DOI:10.34117/bjdv6n10-155

Recebimento dos originais: 05/09/2020
Aceitação para publicação: 07/10/2020

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ABSTRACT
In Brazil, the prevalence of diabetes mellitus (DM) is 11.9 million cases. Diabetic foot ulcers (DFU) increase morbidity and cause hospital admissions among DM patients. In an attempt to better understand DFU, this cross-sectional study investigated microbial content and their susceptibility to antimicrobials. Secretion from foot ulcers of 30 diabetic patients were obtained in three Brazilian hospitals and submitted to microbiological evaluation. All recovered strains were identified and submitted to antimicrobial susceptibility tests. Genetic diversity was investigated by PCR coupled with denaturing gradient gel electrophoresis (PCR-DGGE). DFU exhibited a polymicrobial profile composed of 72.5% aerobic and 22.3% anaerobic bacteria, and 2.5% fungi species. A total of 91 microorganisms were isolated, and the number of recovered species per patient ranged from 1-9.

Peptostreptococcus spp. was the most frequently recovered obligate anaerobic Genus and was resistant mostly to penicillin and clindamycin. A total of 37.5% *S. aureus* strains were methicillin resistant. *E. coli* were the most susceptible Gram-negative species and *Pseudomonas aeruginosa* were the most resistant. The present study demonstrated that almost 34% of microbial species observed on DGGE gels were not cultivable. The recovery of multidrug resistant microorganisms pointed out to the need for more attention when prescribing an empirical therapy and emphasized the relevance of this study.

Keywords: Diabetes mellitus, diabetic foot ulcers, polymicrobial infections, antimicrobial susceptibility, genetic testing.

RESUMO

No Brasil, a prevalència de diabetes mellitus (DM) é de 11,9 milhões de casos. A úlcera do pé diabético aumenta a mortalidade e é causa de internação hospitalar em pacientes com DM. Com objetivo de melhor compreender as lesões do pé diabético, este estudo transversal avalia o conteúdo microbiano e o respectivo perfil de susceptibilidade aos antimicrobianos. A secreção de úlceras nos pés de 30 pacientes foi coletada em três hospitais brasileiros e submetida a análises microbiológicas. Todas as espécies identificadas tiveram seu perfil de susceptibilidade a antimicrobianos testado. A diversidade genética foi avaliada por meio de PCR acompanhada de *Eletroforese em Gel de Gradiente Desnaturante* (PCR-DGGE). As úlceras exibiram um perfil polimicrobiano composto de 72,5% de microrganismos aeróbios, 22,3% anaeróbios e 2,5% fungos. Um total de 91 espécies foram recuperadas sendo de 1 a 9 espécies por paciente. *Peptostreptococcus* spp. foi o gênero de anaeróbios estritos mais frequentemente recuperado e foi resistente principalmente à penicilina e clindamicina. Um total de 37,5% das amostras de *S. aureus* foram resistentes à meticilina. A espécie Gram-negativa mais sensível foi *E. coli* e *Pseudomonas aeruginosa* a mais resistente. O presente estudo mostrou que 34% das espécies microbianas isoladas não foram recuperadas em cultivo. O isolamento de microrganismos multirresistentes realça a necessidade de um maior critério nas prescrições de antimicrobianos de modo empírico e enfatizam a relevância do estudo.

Palavras-chave: Diabetes mellitus, úlcera do pé diabético, infecções polimicrobianas, perfil de susceptibilidade aos antimicrobianos, investigação genética.

1 INTRODUCTION

Diabetes mellitus (DM) is a highly prevalent chronic disease in the human population. Globally, the number of individuals living with DM is expected to exceed half a billion by 2030 [1]. In Brazil, the prevalence of DM is about11.9 million cases [2] and the population ≥30 years of age with type 2 DM is estimated to be at 6.5 million. Between 15 to 25% of patients suffering from DM develop foot ulcers, which are the major cause of morbidity, amputation and hospital admission and corresponded to 9.1-26.1 million patients/year [3, 4]. Diabetic foot ulcers (DFU) is defined as non- or poorly-healing, partial or full thickness wounds, located distal to the ankle in a person with DM [5, 6]. As DFU have lost many protective barriers, they provide a portal for invasive microorganisms [7]. Most acute infections in DM patients not recently treated with antibiotic therapy are monomicrobial and caused predominantly by Gram-positive cocci [8]. In contrast, chronic or previously treated infections are often polymicrobial, typically with the presence of aerobic or

facultative Gram-negative bacilli [5]. Studies also highlighted the importance of strict anaerobes in DFU mixed infections [7, 8] . Antimicrobial resistance has become a growing problem in DFU in developing countries, increasing morbidity, mortality and costs of treatment [9]. Nonetheless, aspects of the microbial community associated to DFU in Brazil, either as causative agents or transitory oppotunists are largely unknown. Considering the scarcity of national and local epidemiologic data in relation to the microbial communities associated with DFU; the misuse of antimicrobials leading to increasing antimicrobial resistance and higher costs of treatment; and the observed drug susceptibility profile of the most prevalent microorganisms recovered as the study progressed the aims of this work were a) to identify the most prevalent microbiota associated to DFUs in patients from public and private hospitals in Belo Horionte, MG; and b) to asses the antimicrobial susceptibility of the cultivable bacteria found in the clinical specimens.

2 MATERIAL AND METHODS

2.1 STUDY DESIGN AND INVOLVED INSTITUTIONS

This was a cross-sectional study involving clinical specimens collected at three Brazilian hospitals: two private institutions and one public university hospital. Clinical samples encompassed tissue, fluid and secretions obtained from foot ulcers of diabetic inpatients. The material was collected during surgery proceedures and analyses were carried out by two independent laboratories: a private laboratory at Belo Horizonte, MG, Brazil; and the laboratory of Oral Microbiology and Anaerobes (at Federal University of Minas Gerais, Belo Horizonte, Brazil).

2.2 PATIENT INCLUSION AND ETHICAL APPROVAL

All individuals in the study population were DM patients requiring hospitalization and presenting DFU, admitted over an one year period. The recent use of antimicrobials did not represent an exclusion criterion. A total of 30 patients were investigated during the study period (from June, 2007 to May 2008). Patient data were obtained from medical records and from interviews with the patient and physician in charge. Previous to sample collection, all patients or responsible patient guardians signed an Informed Consent Form and received orientations concerning their rights. Ethical approval was provided by the Research Ethics Committees of the participating hospitals and by the Federal University of Minas Gerais (ETIC# 391/06).

3 MICROBIOLOGICAL PROCEDURES

3.1 SPECIMEN COLLECTION, TRANSPORT AND IDENTIFICATION

Samples included tissue/secretions and were obtained during surgery procedures and after surgical debridement of devitalized tissues. Specimens were divided into three parts: the first portion was placed in a sterile tube with pre-reduced anaerobically sterilized (PRAS) media for anaerobes under a CO_2 stream [10]. The inoculated media was immediately transported under room temperature to the university laboratory and introduced into an anaerobic chamber (Forma Scientific Co). Next, the tissue was macerated, serially diluted (10^{-2}-10^{-5}) and manually and automatedly tested (WalkAway 96 SI) [9]. The second portion was transported to a private diagnostic laboratory to perform isolation and identification of aerobic bacteria and fungi. Aliquots of 0.1mL of macerated and diluted tissue were spread onto selective and supplemented media [9] and an automated WalkAway 96 SI system was used to identify microorganisms. Sabouraud dextrose agar (SDA) (Difco®) and Mycosel (BD®) agar were used to recover fungi strains [9]. To identify fungi species, microcultivation and WalkAway 96 SI were used. The third tissue portion was aliquoted in sterile Eppendorf® tubes and stored at -86ºC until molecular analyses were employed. All visually distinct colonies in all culture media were identified.

3.2 ANTIMICROBIAL SUSCEPTIBILITY PROFILE

After identification of isolated microorganims, drugs minimal inhibitory concentrations (MIC) were evaluated for all isolates. Anaerobic species were tested through the agar dilution method [10] using penicillin G, amoxicillin/clavulanic acid, metronidazole, meropenem, clindamycin and chloramphenicol. All anaerobic strains were screened for β-lactamase production employing nitrocefin reagent. *Bacteroides fragilis* ATCC 25285 and *Eubacterium lentum* ATCC 43055 were included as validation controls. All aerobic microorganisms had their susceptibility profile investigated by WalkAway® 96 SI. Extended-spectrum β-lactamases (ESbLs) detection was also performed [10]. The *in vitro* activities of amphotericin B, fluconazole and itraconazole were investigated through the E-test method for all isolated fungal species. Results were based on the CLSI M27A-3 [11].

3.3 MOLECULAR ANALYSES

a) Polymerase Chain Reaction - PCR

DNA extraction was performed using phenol-chloroform [12]. PCR reactions targeting the V3 region of RNAr 16S and employing the universal primers 341f (5'-CCTACGGGAGGCAGCAG-3) and 518r (5'-ATTACCGCGGCTGCTGG-3') (Invitrogen, California, USA) were performed

according to Davies et al. [13] with modifications. An additional 40-base-pair GC-rich sequence (GC-clamp) (5'-CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGG-3') was inserted at the 5' end of the 341f primer to enable subsequent experiments. The reaction was performed in a final volume of 25µL containing 50ng of bacterial DNA, 12.5µL of Master Mix (Promega®, Madison, WI, USA) and 25 pmol of each primer. Reaction conditions started with a 95ºC denaturation step (5min), followed by 20 cycles at 94ºC-1 min; 65ºC-45 sec; 72ºC-1min and 10 cycles at 94ºC-1 min; 55ºC-45 sec; 72ºC-1 min. A final extension of 72ºC for 10 min was, then, allowed. Experiments were performed in duplicates and *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 29213, *Bacteroides fragilis* ATCC 25285 and *Prevotella intermedia* ATCC 25611 were used as validation controls. Amplified products were analyzed by agarose gel electrophoresis and were visualized in an UV-transilluminator.

b) Denaturing Gradient Gel Electrophoresis (DGGE)

To investigate genetic diversity, PCR products were separated by DGGE [14], with modifications. A denaturating gradient was created combining 10% polyacrylamide gel and 30-60% of denaturants (urea and formamide) using *Dcode*™ *Universal Mutation Detection* (Bio-Rad, USA). The migration was carried out at 56ºC, 70V (10 min) and then at 170V (3h and 50 min). Gels were stained with SYBR Green (Sigma-Aldrich, USA) and images were obtained in an UV Transilluminator [13]. The same reference strains as described above were used.

4 RESULTS AND DISCUSSION

4.1 CLINICAL CHARACTERISTICS AND PATIENT OUTCOMES

Thirty patients were included in this study, 20 males (66.7%) and 10 females (33.3%). Only one patient was diagnosed with type 1 DM (3.33%), similar to results obtained by Quilici et al. (2016). Infected DFU were detected in 16 patients (53.3%) at some point in their lives and toes were the most common anatomic site of ulcers (20.7%). Other studies [5, 15] also found higher disease prevalence in male patients and toes were also described the most frequent anatomic site of ulcers. Patients assisted in the public hospital represented 80.0% of the study group. A second onset of hospitalization was observed in 40.0% of the patients and the period of hospital stay ranged from 3- to 108 days (mean of 21 days). Conversely, Katz et al. [16] observed that more than half of the studied patients were re-hospitalized and the median length of hospital stay was of 10-65 days. Amputations were performed in 63.4% of patients. During hospitalization, 53.3% of patients were given antimicrobial drugs and none received previous antifungal therapy. This was also similar to the report by Katz et al. [16]. In the present study, changes in antimicrobial prescription were observed after microbial

culture results (in 40.0% of the patients). Initialy amoxicillin/clavulanic acid (27.6%), followed by ceftriaxone and metronidazole (24.1% each) prevailed. Prescriptions changed after hospitalization and meropenem (associated or not with teicoplanin or vancomycin) and vancomycin (always in combination with ceftazidime, piperacillin/tazobactam and/ or meropenem) overcome. New surgical interventions were necessary for 46.7% of study participants and 83.3% of them had to undergo amputation. At the end of the study the mortality rate was of 16.7%, a higher number than that observed by Katz et al. [16] who described a mortality rate of 3% and inferior than that published by Mader et al. [17] who found a mortality rate of 64%.

4.2 MICROBIOLOGICAL RESULTS

Despite previous antimicrobial administration, it was possible to recover viable microorganisms from 93.3% of the patients, and 92.9% of samples came from polymicrobial infections, similarly to previous studies [16]. The number of recovered species per sample ranged from one to nine (mean of four). Other studies [16, 18] also verified the polymicrobial etiology of DFU, and the possibility to recover four to six microorganisms species (mean values) per sample. A total of 91 microorganism isolates were obtained, distributed among 21 genera and including 44 species - 53.7% Gram-negative and 46.3% Gram positive (Table 1).

Table 1: Microbial species isolated from patients with diabetic foot infection.

Types of Microorganisms					
Anaerobes	**n**	**Aerobes**	**n**	**Fungi**	**n**
Prevotella melaninogenica	4	*Staphylococcus aureus*	10	*Candida parapsilosis*	2
Fusobacterium necrophorum	3	*Proteus mirabilis*	9	*Candida albicans*	1
Peptostreptococcus asaccharolyticus	3	*Pseudomonas aeruginosa*	9		
Bacteroides fragilis	3	*Morganella morganii*	8		
Peptostreptococcus magnus	2	*Enterococcus faecalis*	8		
Bacteroides thetaiotaomicron	2	*Escherichia coli*	5		
Peptostreptococcus anaerobius	2	*Enterobacter cloacae*	5		
Acidaminococcus fermentans	2	*Streptococcus agalactiae*	4		
Prevotella bivia	1	*Staphylococcus epidermidis*	4		
Prevotella buccae	1	*Streptococcus milleri* group	3		
Peptostreptococcus anaerobius	1	*Staphylococcus haemolyticus*	2		
Bacteroides uniformis	1	*Staphylococcus warneri*	2		
Porphyromonas gingivalis	1	*Streptococcus GrupoViridans*	2		

Brazilian Journal of Development

Fusobacterium nucleatum	1	*Citrobacter freundii*	2	
		Citrobacter koseri	2	
		Enterococcus avium	2	
		Proteus vulgaris	2	
		Enterococcus faecium	1	
		Alcaligenes spp.	1	
		Staphylococcus simulans	1	
		Providencia stuartii	1	
		Staphylococcus lugdunensis	1	
		Corynebacterium spp.	1	
		Enterococcus raffinosus	1	
		Acinetobacter baumannii	1	
		Acinetobacter lowffii	1	
		Providencia rettgeri	1	
		Staphylococcus hominis-hominis	1	
		Micrococcus spp.	1	
Total	27		91	03

Legend: n - number of isolates.

Anaerobe bacteria were recovered from 15 patients (50%), and similar numbers were obtained by Omar et al. [18]. The most frequently recovered strict anaerobic genus was *Peptostreptococcus* spp. (33.3%) followed by *B. fragilis* and *Prevotella* spp. (22.2% each), and similar findings were reported by Charles et al. [7]. The most frequently recovered aerobic microorganisms were *Staphylococcus* spp. (23.1%), *Enterococcus* spp. (13.2%) and *Proteus* spp. (12.1%), respectively. All patients were under antimicrobial therapy during sampling. Our results are in consonance with other studies that consider *S. aureus* as one of the most common and virulent pathogens recovered from DFUs [6, 19]. Fungal species were recovered from 10.0% of the patients, and *Candida parapsilosis* was the most prevalent. The observed percentage is comparable to those obtained by Bansal et al. [20], whose findings indicated a prevalence of 9.0% of fungal species in DFUs. None of the patients were being administered antifungal therapy at the time of sample collection.

Brazilian Journal of Development

4.3 ANTIMICROBIAL SUSCEPTIBILITY PROFILE

Charles et al. [7] stated that there was no reported antibiotic resistance in anaerobic strains isolated from DFUs in their study. Contrarily, our findings revealed high resistance rates, mostly against penicillin (40.7%) and clindamycin (29.6%), mostly associated to *Bacteroides* spp. (Table 2).

Table 2: Resistance profile of anaerobes recovered from DFU.

	Microorganism	Antimicrobials (n)				
		AMC	CLI	MEN	MET	PEN
Anaerobes (n=27)	*Bacteroides* spp.	00	04	00	00	06
	Prevotella spp.	00	02	00	00	03
	Fusobacterium spp.	00	02	00	00	02
	Peptostreptococcus asaccharolyticus	00	00	00	01	00

Legend: AMC: amoxicillin/clavulanic acid; CLI: clindamycin; MEN: meropenem; MET= metronidazole; PEN: penicillin G.

β-lactamase production was detected in 40.7% of the anaerobic strains. Susceptibility testing of Gram-positive and Gram-negative aerobic strains are presented on Table 3. 37.5% of all recovered *S. aureus* strains were methicillin resistant (MRSA). Smaller rates of MRSA recovering (20.0%) were reported by Omar et al. [18]. *E. coli* was the most susceptible Gram-negative species (60.0% susceptible to 19 tested antimicrobials) and *Pseudomonas aeruginosa* was the most resistant species amongst aerobic isolates (11.1% resistant to 14 of 15 antimicrobials; more than 50% were resistant to β-lactamase). Other authors [18] also reported an increase in the resistance profile of *Pseudomonas aeruginosa* and the presence of multidrug resistance (MDR) among isolates of this species. In our study, it was possible to recover three microorganisms that were β-lactamase producers, including one *E. coli* and two *Proteus mirabilis* isolates. 28.6% of all isolates were β-lactamase inducers. According to Omar et al. [18], β-lactamase inducers among members of the Enterobacteriacea family are an universal concern.

Table 3: Resistance profile of aerobes recovered from DFU.

	Gram negative Microorganism								
Antimicrobials (n)	*Escherichia coli*	*Enterobacter* spp.	*Morganella morganii*	*Proteus* spp.	*Providencia* spp.	*Alcaligenes* sp	*Citrobacter* spp.	*P. aeruginosa*	*Acinetobacter* spp.
AMI	0	0	0	1	0	0	0	1	0
ASB	0	4	6	3	0	0	1	0	0
AMP	2	5	8	6	2	0	4	0	1
AZT	1	1	0	2	0	1	0	4	0

CFZ	1	5	8	5	2	0	2	0	0
CPM	1	0	0	2	0	0	0	4	0
CTX	0	1	0	2	0	0	0	5	0
CAZ	0	1	0	1	0	0	0	4	0
CRO	0	1	0	2	0	0	0	4	0
CFR	1	4	1	4	1	0	0	0	0
CIP	1	0	1	2	1	1	1	3	0
GEN	1	0	0	2	0	0	0	1	0
IMI	0	0	0	0	0	0	0	1	0
LVX	1	0	1	2	1	0	0	3	0
MEN	0	0	0	0	0	0	0	1	0
PTZ	0	0	0	0	0	0	0	4	0
TIC	0	0	0	2	0	0	1	6	0
TO	0	0	0	2	0	0	1	10	0
SMT	1	2	2	3	1	1	1	0	1

Gram positive Microorganism

	S. aureus	*Staphylococcus CoN*	*Enterococcus* spp.
AMC	3	5	0
ASB	3	5	0
AMP	10	9	1
CFZ	3	5	0
CRO	3	5	0
CIP	3	3	2
CLI	3	2	0
ERI	3	4	3
GEN	2	5	0
LVX	1	2	1
LIZ	0	0	0
OXA	4	7	0
PEN	10	8	3
RIF	2	2	5
TET	0	3	5
SMT	1	2	0

Brazilian Journal of Development

VAN	0	0	0
GEN/SIG	0	0	1
STR/SIG	0	0	2

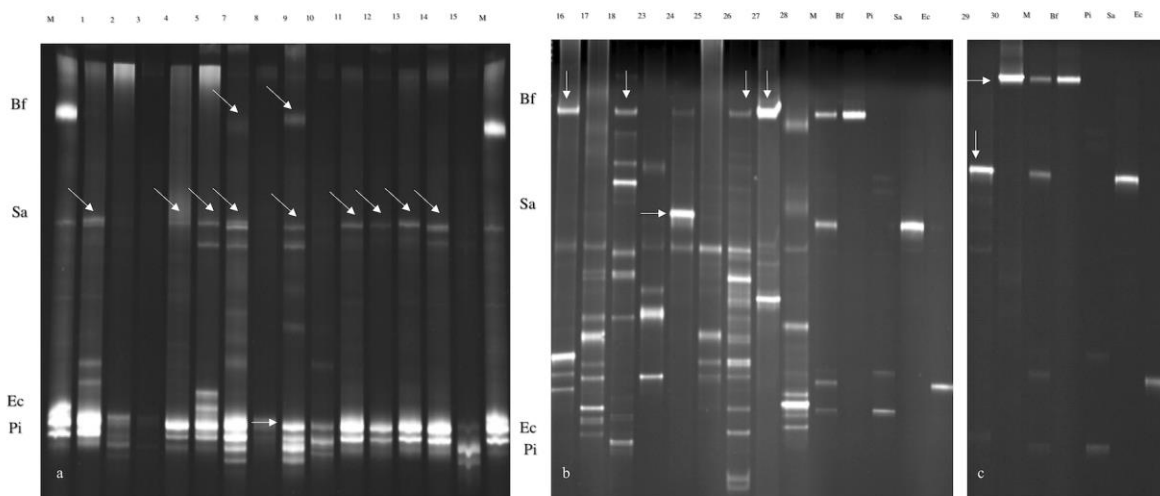
Legend: CoN: coagulase negative; AMI: amikacin; ASB: ampicillin/sulbactam; AMP: ampicillin; AZT: aztreonam; CFZ: cefazolin; COM: cefepime; CTX: cefotaxime; CAZ: ceftazidime; CRO: ceftriaxone; CFR: cefuroxime; CIP: ciprofloxacin; GEN: gentamicin; IMI:imipenem; LVX: levofloxacin; MEN: meropenem; PTZ: piperacillin/tazobactam; TIC: ticarcillin/clavulanate; TO: tobramycin; SMT: sulfamethoxazole.

Of the 12 strains of *Enterococcus* spp. recovered, 16.7% were resistant to penicillin G. Our findings of penicillin G resistance are in accordance to Abdulrazak et al. [15], who found 14% of their samples resistant to the same antimicrobial drug. All fungal species recovered here were susceptible to all antifungals tested. One sample of *S. aureus* and one of *P. mirabilis* were resistant to 64.7% and 78.9% of all tested antimicrobials. Importantly, these were recovered the same patient. The recovery of several drug-resistant strains, including anaerobes and multidrug resistance bacteria, stress the need to promote the rational use of antimicrobial inside healthcare institutions. Likewise, our results emphasize the importance to perform local epidemiological surveillance to minimize therapeutic failures.

4.4 PCR FOLLOWED BY DENATURING GRADIENT GEL ELECTROPHORESIS (PCR-DGGE)

The use of PCR-DGGE makes it possible to investigate many DFU samples from a number of patients in a reduced amount of time. Additionally, the method could facilitate the indentification of fastidious and uncultivable pathogens in healthcare facilities that do not posses access to metagenomic approaches and next generation sequencing capabilities.

Figure 1- DGGE profile of clinical specimens recovered from DFU, using universal primers



Brazilian Journal of Development

Legend: a: Patients 1 to 5 and 7 to15. White arrows: *B. fragilis (*Patients 16, 18, 26 and 27) and *S. aureus (*Patients 1, 4, 5, 7, 9 and 11-14); b: Patients16 to18 and 23 to 28. White arrows: *B. fragilis (*Patients 7 and 9) and *S. aureus (*Patient 24); c: Patients 29 and 30. White arrows: *B. fragilis* (Patient 30) and *S. aureus (*Patient 24). M: mixture of reference samples. Bf: *Bacteroides fragilis* ATCC 25285, Pi: *Prevotella intermedia* ATCC 25611, Sa: *Staphylococcus aureus* ATCC 29213, Ec: *Escherichia coli* ATCC 25922.

Our results indicated that almost 34% of microbial species detected by PCR-DGGE were not cultivable. Samples recovered from Patients 1-5 and 7-15 are depicted in Figure 1-a (median of six bands per lane). PCR-DGGE analysis from patients 2 and 3 revealed some bands and the consequent presence of microbial DNA, whilst classical methods did not reveal any cultivable microorgnaism. The differences between methods could be attributed to previous antimicrobial intake and to limitations of culture techniques, reinforcing the importance of molecular methods. A band corresponding to *S. aureus* was observed for Patients 1, 4, 5, 7, 9 and 11-14. However, microbiological culture was positive only for Patients 5, 12 and 13. Bands corresponding to *P. intermedia* and *E. coli* were observed for almost all patients, and to *B. fragilis* only for Patients 7 and 9. Figure 1-b shows the results for Patients 16-18 and 23-28. Those lanes exhibited more bands (median of nine per patient) than in part ‘a’ of the figure. Samples from Patient 24 revealed one strain of *S. aureus* concomitantly detected by PCR-DGGE and by culture, whereas *B. fragilis* was detected only by the molecular technique. *B. fragilis* was also detected in Patients 26 and 27 by classical and genetic methods. Samples from Patients 16 and 18 revealed non-*Bacteroides* spp. *fragilis* isolates in culture and a band corresponding to *B. fragilis* was observed by PCR-DGGE. At last, samples from Patient 30 (Figure 1-c) resulted in the absence of growth in culture, but a band corresponding to *B. fragilis* DNA was detected in the PCR-DGGE analysis. The presence of *S. aureus* on Patient 29 was detected by culture and PCR. Waters et al. [21] also reported low sensitivity of culture methods and the difficulty to cultivate obligate anaerobes.

5 CONCLUSIONS

Taken together, ou results showed that *Peptostreptococcus* spp. was the most frequently observed strict anaerobic Genus recovered from DFU patients, whereas 37.5% of all recovered *S. aureus* strains were methicillin resistant. *Pseudomonas aeruginosa* were the most drug-resistant Gram-negative species and, on the other hand, *E. coli* was the most susceptible gran-negative bacteria obtained from the patients’ tissues. The present study demonstrated that almost 34% of all microbial species detected by PCR-DGGE were not cultivable, which highlights the difficulties that physicians face when prescribing empirical drug therapies. It is important to emphasize that despite the current application of metagenomic approaches on clinical practices, these are not widely available,

especially in underdeveloped regions. Thus, simpler and more affordable strategies like the PCR-DGGE may still represent a valuable alternative in such regions.

Declarations

• Funding:

This work was supported by Conselho Nacional de Desenvolvimento Científico e Tecnológico - CNPq, Fundação de Amparo à Pesquisa do Estado de Minas Gerais - FAPEMIG and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior- CAPES.

• Conflict of Interest:

Authors declare no conflict of interest.

• Ethical approval:

Ethical approval was provided by the Research Ethics Committees of the participating hospitals and by the Federal University of Minas Gerais (ETIC# 391/06).

• Informed consent:

The authorization for inclusion was obtained by signing the patients or legal guardians of the Informed Consent Term (TCLE).

ACKNOWLEDGEMENTS

We thank those patients who voluntarily participated in this study. This work was supported by Conselho Nacional de Desenvolvimento Científico e Tecnológico - CNPq, Fundação de Amparo à Pesquisa do Estado de Minas Gerais - FAPEMIG and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior- CAPES.

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