Short report

Polymerase chain reaction–denaturing gradient gel electrophoresis (PCR–DGGE): A promising tool to diagnose bacterial infections in diabetic foot ulcers

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Abstract

Aim. – The diagnosis of diabetic foot infections is difficult due to limitations of conventional culture-based techniques. The objective of this study was to evaluate the contribution of denaturing gradient gel electrophoresis (DGGE) in the microbiological diagnosis of diabetic foot ulcers in comparison to conventional techniques, and also to evaluate the need to perform a biopsy sample for this diagnosis.

Methods. – Twenty diabetic patients (types 1 and 2) with foot ulcers (grades 1–4) were included. After debridement of their wounds, samples were taken in duplicate by surface swabbing and deep-tissue biopsy. The samples were analyzed by conventional culture and by a new molecular biology tool, DGGE technology.

Results. – Polymerase chain reaction (PCR)–DGGE led to the identification of more bacteria than did conventional cultures (mean: 2.35 vs 0.80, respectively). In 11 cases, the technology detected pathogenic species not isolated by classical cultures. PCR–DGGE also identified significantly more pathogenic species at deep levels compared with species detected at superficial levels (87% vs 58%, respectively; P = 0.03). In 9/20 cases, pathogenic bacteria were detected only in deep samples, revealing the need to perform tissue biopsy sampling.

Conclusion. – DGGE, achievable in 48 h, could be a useful technique for the bacteriological diagnosis of diabetic foot infections. It may help to identify pathogenic bacteria in deeply infected ulcers, thereby contributing to a more appropriate use of antibiotics.

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1. Introduction

Foot ulcers are common in diabetic patients with prevalence as high as 25% [1], but due to the confounding effect of neuropathy and ischaemia on local and systemic inflammatory responses, diagnosing foot infection at an early stage in diabetic individuals is often difficult [2]. Reliable identification of clinical and/or microbiological criteria for diabetic foot infection would be of great value, as it would allow the best documentation of diabetic foot ulcers (DFUs) and the most appropriate antibiotic treatment. However, up to now the only microbial diagnostic tool available has been routine clinical cultures, but this technique does not necessarily select the most abundant or clinically important organisms. It is also limited to only fastidious bacteria and microorganisms organized into biofilms. Denaturing gradient gel electrophoresis (DGGE) is a well-established tool for molecular microbial ecology. This useful new technique allows comparisons among the diverse

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microbial communities and monitoring of bacterial population dynamics. The present pilot study has evaluated the contribution of DGGE in the microbiological diagnosis of DFUs compared with conventional techniques.

2. Methods

2.1. Prospective pilot study

Twenty outpatients attending a French foot clinic (University Hospital Nîmes) for any type of foot ulcer were prospectively enrolled between 4 February 2008 and 17 April 2008 after their informed consent was obtained. This study was approved by the local ethics committee (South Mediterranean III) and carried out in accordance with the Declaration of Helsinki as revised in 2000. Every patient was examined by a trained physician to grade infection severity, and all antibiotic treatments were noted. According to the Infectious Diseases Society of America (IDSA)/International Working Group on the Diabetic Foot (IWGDF) criteria [3], wounds were considered either uninfected (grade 1) or infected (grade ≥ 2). Although samples from grade 1 ulcers are not usually taken as a clinical routine, they were performed for this study to allow comparisons of conventional culture and DGGE in all situations. Using the University of Texas (UT) classification, ulcers were distinguished as either superficial or deep [4].

2.2. Study design

After wound debridement, samples for bacterial culture and molecular biology were obtained by swabbing the wound and by taking deep-tissue biopsies (n = 40). The samples were frozen at -20°C and immediately sent to the bacteriology department.

2.3. Microbiological study

Part of each sample (one swab and one biopsy per patient) was cultured on usually agar media. For each colony isolated, genus and species were determined using the VITEK 2 card system (bioMérieux France, Craponne). Pathogenic bacteria comprised Staphylococcus aureus, β-haemolytic streptococci, enterobacteria and anaerobes, whereas commensal flora included cutaneous bacteria (coagulase-negative staphylococci, corynebacteria and Acinetobacter spp.). Intermediate flora included low-virulence bacteria such as Pseudomonas aeruginosa, Enterococcus spp. and Stenotrophomonas maltophilia.

2.4. Polymerase chain reaction (PCR)–DGGE

The remainder of each sample (one swab and one biopsy per patient) was cultured on usually agar media. For each colony isolated, genus and species were determined using the VITEK 2 card system (bioMérieux France, Craponne). Pathogenic bacteria comprised Staphylococcus aureus, β-haemolytic streptococci, enterobacteria and anaerobes, whereas commensal flora included cutaneous bacteria (coagulase-negative staphylococci, corynebacteria and Acinetobacter spp.). Intermediate flora included low-virulence bacteria such as Pseudomonas aeruginosa, Enterococcus spp. and Stenotrophomonas maltophilia.

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The DNA of each sample was extracted by using MasterPure DNA Purification Kits (Epicentre Biotechnologies, Madison, WI, USA) in accordance with the manufacturer’s instructions. The V3 region of the 16S rRNA gene was amplified (with additional GC clamp) using eubacterium-specific primers HDA1 (5′-CGGCCCGCGC-GCGGCGGGCGGCGGGCGGAGCTGCTAC-3′; V3F-GC) and W104 (5′-TTACCGCGCTGCTGGCAGC-3′; V3R) [5]. The PCR mixture in the final volume of 25 μL comprised 5 μL of 5X KAPA2G buffer (containing 1.5 mM MgCl2), 200 μM of dNTP, 0.5 μM of each primer, 0.5 units of KAPA2G Fast HotStart DNA polymerase and 1 μL of template DNA. Reactions were performed in 0.5-mL DNA-free PCR tubes using a thermal cycler (Eppendorf France S.A.S., Le Pecq), and the PCR steps were as follows: 95°C (2 min), followed by 30 cycles each at 95°C (15 s), 61°C (15 s) and 72°C (2 s), and a final cycle at 72°C (5 min).

DGGE migration was carried out using the CBS-DGGE 2000 system (C.B.S. Scientific Co., San Diego, CA, USA). Polyacrylamide gel electrophoresis was carried out at 90 V at a constant 60°C for 18 h. All gels were stained for 1 h using SYBR Gold stain (Invitrogen, Carlsbad, CA, USA) and photographed using the Molecular Imager Gel Doc XR System (Bio-Rad, Marnes-la-Coquette, France). Gel images were analyzed using FingerPrint 2 software. Dendrograms were constructed by cluster comparisons using the Pearson coefficient [6].

2.5. Sequencing the DGGE bands and analysis

DGGE bands were excised using a sterile scalpel, then placed in nuclease-free tubes with 200 μL of elution Buffer EB (Qiagen, Hilden, Germany) and incubated for 3 h at 37°C. Extracts (2 μL) were used as templates for PCR sequencing. After PCR product purification, the sequencing was carried out using a PerkinElmer ABI 377 sequencer. Sequence analyses were performed using SeqScape software (Applied Biosystems, Foster City, CA, USA). Sequence matching was undertaken using National Center for Biotechnology Information (NCBI) databases (www.ncbi.nlm.nih.gov/blast) to identify the species.

2.6. Statistical analysis

The presence of pathogenic/non-pathogenic species was compared according to depth of samples using Fisher’s exact test. Statistical analysis was performed using S-Plus 2000 software (Insightful Corp., Seattle, WA, USA), and the results were considered significant at P < 0.05.

3. Results

3.1. Clinical data

During the study period, 20 patients were recruited. Most of the included patients were male (75%) and aged > 60 years; 90% had type 2 diabetes (HbA1c median: 8.3%). In the year prior to hospitalization, 60% of patients had a history of infected foot ulcer and, at presentation, 10% had had a previous lower-limb amputation at the toe level. Two wounds (10%) were classified as uninfected (grade 1) and 18 (90%) as infected (grades 2 to 4). Seven ulcers were superficial and 13 were deep (Fig. 1).

3.2. Construction of a DGGE diversity ladder

With the aim of adapting DGGE into a routine diagnostic tool, 14 species were selected (Bacteroides fragilis, Clostridium perfringens, Enterococcus faecalis, Enterobacter aerogenes, Escherichia coli, Klebsiella pneumoniae, Propionibacterium acnes, Staphylococcus aureus, Staphylococcus epidermidis,
Fig. 1. Results obtained with PCR–DGGE vs conventional bacterial culture.

Streptococcus agalactiae, Proteus mirabilis, Pseudomonas aeruginosa, Streptococcus pneumoniae and Streptococcus pyogenes) to construct a reference ladder on the basis of their prevalence in DFUs and the diversity of their migration patterns across the gel.

3.3. Bacterial ecology with PCR–DGGE and bacterial culture of superficial and deep samples

Fig. 1 shows the bacterial ecology obtained by both PCR–DGGE and bacterial culture. With PCR–DGGE, 47 species were isolated from 20 samples, with a mean of 2.35 ± 1.6 isolates per sample. Polymicrobial infection was present in 16 samples (80%). One grade 1 ulcer was negative, while only one bacterial species was isolated on the other grade 1 wound. Staphylococcus aureus was the most commonly isolated pathogen (28.6% of all aerobic bacteria, 44.6% of all Gram-positive cocci). Conventional culture identified exclusively 16 species (mean: 0.8 ± 1.5), and was positive in only 65% of the deep samples. Aerobic Gram-positive cocci were predominant (62.5% of all pathogens). Seven patients (35%) had negative samples. Thus, in terms of bacterial ecology, our study showed
that PCR–DGGE identified more bacteria than did conventional cultures.

3.4. Identification of pathogenic and non-pathogenic bacteria

Comparison of the two techniques indicated that, in 11 out of 20 (55%) biopsies, DGGE identified pathogenic species not isolated by conventional culture (Fig. 1). In six biopsy samples (C1P01, C1P14, C1P15, C1P16, C1P20, C1P22), no species was detected by conventional culture, whereas PCR–DGGE identified at least one pathogenic species (β-haemolytic streptococci, S. aureus and/or Enterobacteriaceae). In five other cases (C1P06, C1P10, C1P19, C1P21, C1P23), PCR–DGGE led to the detection of at least one pathogenic species (anaerobes, S. aureus and Proteus mirabilis) in addition to those found by cultures. In only seven (35%) deep samples, pathogenic species were detected by both PCR–DGGE and culture. Interestingly, anaerobes—pathogens difficult to identify—were detected in 10.6% of all isolates (40% of deep DFUs) by PCR–DGGE, whereas these pathogens were absent in conventional cultures.

Thus, in terms of distinguishing between pathogenic and non-pathogenic bacteria, PCR–DGGE identified a considerably greater number of pathogenic bacteria than did conventional culture.

3.5. Superficial and deep bacterial flora

At the deep level, PCR–DGGE identified significantly more pathogenic species (20/23, 87%) compared with the same analysis at the superficial level (18/31, 58%; \( P = 0.03 \); Fig. 1). On the other hand, PCR–DGGE identified significantly more commensal bacterial species at the superficial level (13/31, 41.9%) than at the deep level (2/23, 8.7%; \( P = 0.01 \)). In nine cases out of 20, pathogenic species were found only in deep samples and not in surface samples, thus demonstrating the usefulness of deep samples in the diagnosis of DFU infections. Moreover, in these nine cases, the antibiotic treatment was not appropriate for the true microbial ecology present in the wound (Fig. 1).

Thus, PCR–DGGE identified a considerably greater number of pathogenic bacteria in deep samples than did conventional techniques, and confirmed the low value of superficial samples in cases of deep DFU. In addition, the technique enabled optimized antibiotic treatment.

4. Discussion

The results of the present pilot study have highlighted DGGE as a useful technique for the bacteriological diagnosis of diabetic foot infections. Optimal treatment of such infections requires recognizing which ulcers are infected and prescribing pathogen-appropriate antibiotic therapy. This implies that microbiological diagnoses are currently a challenging problem. It is known that the limitations of culture-based techniques include the biased selection of species that flourish under the typical nutritional and physiological conditions of the diagnostic microbiology laboratory, and do not necessarily reveal the most abundant or clinically important organisms [7]. Over the past decade, studies using molecular techniques have raised doubts as to the accuracy of wound culture results [8–14]. Indeed, the technologies have revealed vastly more complex bacterial communities than those identified by culture.

In our study, we developed DGGE as a routine diagnostic tool. It is a simple technique that does not require either complex training or bioinformatics expertise and is inexpensive in terms of consumables (≈ 15 € per analysis vs ≈ 7 € for analysis by cultures). In under two days (vs a minimum of five days for cultures), results were obtained that allowed the development of a reference ladder, thus avoiding the need for sequencing. This affordable technology could ultimately replace the increasingly antiquated culture methods currently used in bacteriology laboratories. Other techniques in bacterial diagnostics have also emerged, such as matrix-assisted laser desorption/ionization mass spectrometry (MALDI-TOF MS) [15] and pyrosequencing [13,14]. However, MALDI-TOF MS still uses bacterial colonies grown on agar media, a major problem in DFU because, as mentioned above, certain bacteria are underrepresented in such traditional cultures, thereby limiting the value of MS at this time, while pyrosequencing requires high-cost equipment and technician time, and an important delay in reporting results [12,14].

Our use of DGGE has confirmed several points: cultures greatly underestimate wound flora while overestimating the relative abundance of staphylococci; and the prevalence of obligate anaerobes is underrepresented. Furthermore, tissue specimens are more accurate than swabs for bacteriological analysis. Moreover, DGGE may help clinicians to optimize antibiotic therapy against pathogens (more appropriate treatment could be given in 45% of cases).

The main limitation of DGGE concerns the technique itself. It underestimates approximately 20% of the bacterial flora, notably anaerobes (an estimated 30% of wounds) [16]. Nevertheless, DGGE was able to detect a large number of pathogenic species and improved the clinical management of those wounds, as previously noted [17]. However, the condition of the samples and their transport remain crucial for the cultivation of anaerobes, regardless of the techniques used.

The development of molecular microbiological technologies is a promising step towards a better understanding of the local ecology of DFUs. It may ultimately help clinicians by optimizing antibiotic therapy against the actual pathogens present in such wounds.

Disclosure of interest

The authors declare that they have no conflicts of interest concerning this article.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.diabet.2014.03.002.

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