Distinct polymicrobial populations in a chronic foot ulcer with implications for diagnostics and anti-infective therapy

Can Imirzalioglu, Shneh Sethi, Christian Schneider, Torsten Hain, Trinad Chakraborty, Peter Mayser, and Eugen Domann

Abstract

Background: Polymicrobial infections caused by combinations of different bacteria are being detected with an increasing frequency. The evidence of such complex infections is being revealed through the use of novel molecular and culture-independent methods. Considerable progress has been made in the last decade regarding the diagnostic application of such molecular techniques. In particular, 16S rDNA-based sequencing and even metagenomic analyses have been successfully used to study the microbial diversity in ecosystems and human microbiota. Here, we utilized denaturing high-performance liquid chromatography (DHPLC) as a diagnostic tool for identifying different bacterial species in complex clinical samples of a patient with a chronic foot ulcer.

Case presentation: A 45-year-old female suffered from a chronic 5x5cm large plantar ulcer located in the posterior calcaneal area with subcutaneous tissue infection and osteomyelitis. The chronic ulcer developed over a period of 8 years. Culture and DHPLC revealed a distinct and location-dependent polymicrobial infection of the ulcer. The analysis of a superficial biopsy revealed a mixture of Staphylococcus aureus, Proteus vulgaris, and Fusobacterium nucleatum, whereas the tissue-deep biopsy harbored a mixture of four different bacterial species, namely Gemella morbillorum, Porphyromonas asaccharolytica, Bacteroides fragilis, and Arcanobacterium haemolyticum.

Conclusions: This clinical case highlights the difficulties in assessing polymicrobial infections where a mixture of fastidious, rapid and slow growing bacteria as well as anaerobes exists as structured communities within the tissue architecture of chronic wound infections. The diagnosis of this multilayered polymicrobial infection led to a microbe-adapted antibiotic therapy, targeting the polymicrobial nature of this infection in addition to a standard local wound treatment. However, a complete wound closure could not be achieved due to the long-lasting extensive destruction of tissue.

Keywords: Polymicrobial infection, Foot ulcer, Microbiome, Gemella morbillorum

Background

Polymicrobial diseases caused by combinations of different bacteria, as well as viruses, fungi, and parasites are being detected with an increasing frequency [1,2]. There are different ecological features underlying the induction of polymicrobial diseases, such as microbial interference, where (i) one microorganism generates a niche in the host that suppresses the colonization of other microorganisms or synergistic polymicrobial infections, where (ii) one microorganism generates a niche favorable for the infection and colonization of other microorganisms, or where (iii) one microorganism predisposes the host to colonization by other microorganisms as evidenced between some periodontal pathogens [2,3]. Another example of a polymicrobial infection is an additive infection implying that two or more microorganisms can synergistically cause infection as seen in different clinical entities, such as bacteraemia, abdominal abscess, secondary peritonitis [4], soft-tissue infection or fasciitis [5]. Furthermore, many bacteria are able to form biofilms which provide a perfect niche, a
Achilles tendon was also affected resulting in a destruction almost the entire tuber calcanei by confluent foci. The osteomyelitis was space affecting confirmed the suspected diagnosis of osteomyelitis calcaneal bone, originating from the posterior, plantar examination revealed inflammatory destruction of the wound area revealed a granulomatous inflammation and showed signs of chronic tissue-biopsies from the wound area revealed a granulomatous inflammation and showed signs of chronic osteomyelitis was suspected due to the deep lomatous inflammation and showed signs of chronic histological examination, because of the long lasting situations of ulcers and osteomyelitis are frequently observed in chronic diabetic foot infections, an assessment of possible underlying endocrinological causes was carried out. Here, no clinical evidence for diabetes or metabolic syndrome could be found as, among others, HbA1c as a diagnostic marker was unremarkable and blood sugar levels were normal (Table 1). The ulcer also was off odors and obviously impaired her social life. A neurologic evaluation revealed a chronic, idiopathic polyneuropathy, resulting in a sensory impairment also involving pain perception. The suspected reason for the polyneuropathy was a previous herniated disc which also remained untreated. Furthermore, a generalized anxiety disorder was diagnosed which prompted the patient to evade consultations.

For microbiological analysis we obtained both a superficial and a deep subcutaneous tissue biopsy for cultural and molecular examinations (Figure 1A). The samples were routinely processed for culture and for PCR. Briefly, the biopsies were homogenized by using the FastPrep Cell Disrupter (MP Biomedicals, Germany) and firstly streaked on MacConkey, blood, chocolate, Schaedler, and Sabouraud agar plates. The agar plates were incubated at 37°C for two days except for Schaedler and Sabouraud agar plates which were incubated for two weeks and four weeks, respectively. Additionally, an enrichment culture was done in thioglycollate. Secondly, the homogenized samples were subjected to nucleic acid extraction, PCR, and subsequent denaturing high-performance liquid chromatography (DHPLC) as described [7]. DHPLC is a technique proven to be appropriate for the molecular detection of polymicrobial infections. It is capable of separating mixed PCR amplicons derived from a mixture of bacteria in a sample by using a cartridge with microspheres, a fragment collector, amplicon sequencing, and bioinformatics [7-11].

Since the patient was in Yucatán/Mexico when she sustained the injury a Bairnsdale or Buruli ulcer caused by Mycobacterium ulcerans was suspected. But both microscopy for acid-fast bacteria and a Mycobacterium-specific PCR performed from the biopsies were negative. A culture performed on Loewenstein-Jensen at 32°C for 4 months also remained negative. The search for a chronic subcutaneous mycosis, an important differential diagnosis for the patient’s clinical picture, could be excluded by performing fungal culture, dermatohistopathology (PAS, Calcofluor white, Fontana-Masson and Gomori stain), and pan-fungal PCR which all remained negative. However, we did find distinct polymicrobial populations in a superficial and a tissue-deep biopsy which did not overlap. The culture-analysis of the superficial biopsy revealed a mixture of Staphylococcus aureus, Proteus vulgaris, and Fusobacterium nucleatum which was obtained.
by culture and confirmed by 16S rDNA PCR by using DHPLC. For the tissue-deep biopsy no growth of bacteria using different culture conditions as described above could be obtained. However, these samples were positive for 16S rDNA PCR. Subsequent DHPLC-based analysis permitted the identification of four different bacterial species in the sample, namely Gemella morbillorum, Porphyromonas asaccharolytica, Bacteroides fragilis, and Arcanobacterium haemolyticum. DHPLC also enables the quantification of the amount of bacterial rDNA derived from individual species [7]. Therefore, the amount of bacteria per biopsy (~10 mg tissue) was calculated for S. aureus as ~10^2, for P. vulgaris as ~10^3, for F. nucleatum as ~10^3, for G. morbillorum as ~5x10^6, for P. asaccharolytica as ~8x10^4, for B. fragilis as ~5x10^4, and for A. haemolyticum as ~5x10^4 in the samples (Figures 1C and 2).
G. morbillorum was identified to be the predominant species in this structured chronic wound infection. It is a gram-positive coccus and a member of the commensal organisms located on the mucous membranes of humans and warm-blooded animals [12]. However, a number of reports have suggested that Gemella can cause both severe localized and generalized infections. These infections include abscesses [13,14], endocarditis [15-18], endovascular infections [19,20], infections of the central nervous system [21], septic arthritis [22], and skeletal infections [23]. Gemella spp. possesses a typical gram-positive cell wall structure. However, during Gram staining cells easily lose their color and may therefore appear gram-negative (gram-labile). This could probably be the reason for frequent misidentification and the low number of clinical case reports associated with Gemella infections. Rapid identification systems based on biochemical features or MALDI-TOF mass spectrometry require cultured bacteria and do not offer reliable identification of all strains of these species once cultivated [24-26].

All of the other bacteria were detected in significantly lower amounts (Figure 1): From the deep-tissue biopsy P. asaccharolytica, B. fragilis, and A. haemolyticum were isolated, which have all been reported to be capable of causing chronic cutaneous or even necrotizing abscesses [27,28]. From the superficial biopsy, which represented the upper level of the wound the clinically well-known and frequently isolated bacteria S. aureus, P. vulgaris, and F. nucleatum were detected. All of these bacteria are known to cause severe wound infections and even necrotizing abscesses [27,29,30].

Interestingly, there were no similarities in bacterial species from either sample suggesting two distinct polymicrobial populations, one located superficially and one located in the deep subcutaneous tissue. Therefore, we postulated that a polymicrobial infection – although not necessarily the primary cause - with predominance of G. morbillorum aggravated the lesion, and thereby complicated the healing process. The diagnosis of this multi-layered polymicrobial infection prompted a local wound treatment with AQUACELL™ Ag (Convatec GmbH, Munich, Germany) and Mepilex® Lite (Mölnlycke Health Care GmbH, Vienna, Austria). AQUACELL™ Ag is based on an advanced Hydrofiber® technology which locks in fluid and traps bacteria. Incorporated ionic silver exhibits antimicrobial activity and kills a wide spectrum of wound associated bacteria. Mepilex® Lite consists of a thin flexible sheet of absorbent hydrophilic polyurethane foam which is designed for the management of a wide range of exuding wounds such as leg and foot ulcers and which provides a moist wound-healing environment. Furthermore, the diagnosis enabled a microbe-adapted antibiotic therapy, targeting the polymicrobial nature of this infection with emphasis on the predominant pathogen G. morbillorum (based on previously reported Gemella resistance traits), with clindamycin (daily dose: 3×600 mg) and levofloxacin (daily dose: 1×500 mg) over a period of 6 weeks. Although the ulcer was profuse, deep, and already persisting for nearly a decade the induced treatments prevented further dissemination of the infection, eliminated the potential threat of amputation, and supported tissue regeneration. However, a complete wound closure could not be achieved during the course of this study due to the long-lasting extensive destruction of tissue.

Chronic skin and soft tissue ulcers present challenges regarding diagnosis and treatment. These wounds are frequently caused by a diverse mixture of bacteria

### Table 1. Laboratory tests performed upon consultation

| Tests                              | Values       |
|------------------------------------|--------------|
| **Hematology**                     |              |
| Leukocytes [tera/l] Eb             | 16.8 +       |
| Erythrocytes [tera/l] Eb           | 5.0          |
| Hemoglobin [g/l] Eb                | 132          |
| Hematocrit [l/l] Eb                | 0.40         |
| Thrombocytes [tera/l] Eb           | 343          |
| MCV [fl] Eb                        | 80           |
| MCH [pg] Eb                        | 26.6 +       |
| MCHC [g/dl] Eb                     | 33.4         |
| Neutrophils [tera/l] Eb            | 11.30 +      |
| Eosinophils [tera/l] Eb            | 0.30         |
| Basophils [0.03] Eb                | 0.03         |
| Lymphocytes [tera/l] Eb            | 3.06         |
| Monocytes [tera/l] Eb              | 1.17 +       |
| Erythrocytes sedimentation rate [mm/H] Eb | 48/81      |
| HbA1c [%] Eb                       | 6.0          |
| **Clinical chemistry**             |              |
| Sodium [mMol/l] Hp                 | 140          |
| Potassium [mMol/l] Hp              | 4.1          |
| Creatinine [mg/dl] Hp              | 0.6          |
| Urea [mg/dl] Hp                    | 28           |
| Uric acid [mg/dl] Hp               | 5.2          |
| Protein [g/l] Hp                   | 81           |
| LDH [U/l] Hp                       | 256 +        |
| GOT (AST) [U/l] Hp                 | 22           |
| GPT (ALT) [U/l] Hp                 | 20           |
| GGT [U/l] Hp                       | 41 +         |
| **Plasma proteins**                |              |
| CRP [mg/l] Hp                      | 60.1 +       |
| IgE [IU/ml] Hp                     | 36.1         |
| Antistreptolysin [IU/ml] Hp        | 148          |

Deviations from the standard values are indicated with a plus (+) sign for increased and a minus (-) sign for decreased values. Both are highlighted in bold type.
encompassing fastidious, rapid and slow growing microbes as well as anaerobes. The traditional detection of such diverse microbes by culture requires dedicated media and culture conditions and is frequently unsatisfactory although the conditions used were appropriate. Therefore, many laboratories also use molecular methods such as PCR to detect pathogens involved in polymicrobial infections. The combination of traditional culture methods and molecular techniques enhances pathogen detection and therefore enables a pathogen-adapted anti-infective therapy. Interestingly, the bacterial community in the superficial biopsy could be cultivated and also detected via PCR. However, the microorganisms in the tissue-deep biopsy were nonculturable. Therefore, the polymicrobial nature of this tissue-deep biopsy was only detectable by culture-independent, molecular methods (PCR and DHPLC). It is known that anaerobes die quickly after sample collection and transport into the laboratory and that diagnosis by culture is therefore difficult [31]. But in our case the transportation times to the laboratory were short and the samples were immediately processed. One would expect that G. morbillorum and A. haemolyticum were cultivable since the first one is a microaerophilic bacterium, although rather slow growing, and the second one is facultative anaerobe. We hypothesize that the reason for the failure of the culture lies in the potential presence of a polymicrobial biofilm in the deep wound where the microbes existed in a viable but nonculturable (VBNC) status [32,33]. Furthermore, discrepancies between superficial and deep wound samples are often observed in diabetic foot infections, as a model for chronic wound situations, as stated in several publications [34-36]. However, in some of these cases the deep sample or even the bone biopsy remained negative despite clinical evidence of infection. Unfortunately, these samples were not evaluated by molecular methods, as a comparable situation as in this case could be the underlying cause of the culture negativity.

Conclusions
This clinical case highlights the difficulties in assessing polymicrobial infections where a mixture of fastidious, rapid and slow growing bacteria as well as anaerobes exists as structured communities within the tissue architecture of chronic wound infections. The evidence of such complex infections is being revealed through the

Figure 2 Standard curve to quantify the amount of bacteria in the biopsies. The standard curve was generated with Pseudomonas aeruginosa as described [7].
use of novel molecular and culture-independent methods. Here, subjecting biopsies obtained from various regions of the wound to DNA-based detection methods provides additional information not available by conventional culture methods. The identification of *G. morbillorum* as a predominant bacterial species associated with the pathogenesis of the wound infection would have probably been missed without the use of an additional molecular technique. Considerable progress has been made in the last decade regarding the diagnostic application of molecular techniques. In particular the use of the 16S rDNA-based sequencing and even metagenomic analyses have been successfully used to study the microbial diversity in ecosystems and human microbiota [37–40]. However, these approaches require sophisticated instrumentation and technologies employing next-generation sequencing machines with subsequent bioinformatic analyses of metagenomic data [41, 42]. At least two hurdles must be overcome for the implementation of these techniques in the clinical diagnostic laboratory. Firstly, these techniques are still time consuming, difficult to reproduce, not yet standardized, and labor-intensive, thereby hindering their routine use in a diagnostic laboratory. Secondly, data linking detection of polymicrobial infection and their utility in assessing clinical states is largely missing [42–44]. Here, we utilized DHPLC as an additional diagnostic tool (staged diagnostics) for identifying different bacterial species in a complex clinical sample. This technology not only allows resolution of mixed amplicons quantitatively, but also permits subsequent sequencing of the individually separated amplicons [7,9]. Falling costs in high-throughput next-generation sequencing technologies and the implementation of enhanced bioinformatic tools, to enable “next-generation diagnostic capabilities” will considerably improve the detection potential of polymicrobial infections in the standard clinical microbiology laboratory.

**Consent**

Written informed consent was obtained from the patient for publication of this Case report and any accompanying images. A copy of written consent is available for review by the Editor of this journal. The study was approved by the Ethics Board of the Justus-Liebig-University of Giessen.

**Competing interests**

The authors declare that they have no competing interests.

**Authors’ contributions**

CI and ED did the microbial examinations, drafted and wrote the manuscript and generated the figures. PM took care of the patient, supervised the clinical guidance and prompted along with CS the medical examinations. Both contributed to the conception of the manuscript and the discussion. SS and TC participated in the conception and writing of the manuscript. TH supervised the sequencing and bioinformatics of the PCR products and contributed to the discussion. All authors read and approved the final version of the manuscript.

**Acknowledgements**

The authors thank the patient for her consent to publish the case and Silke Zechel-Gran and Kirsten-Susann Bommersheim for excellent technical assistance.

This work was supported by the research grant “Metagenomics and host-pathogen interactomics in diabetic foot infections” to E.D. and T.C. (FKZ: 0315880C) from the Bundesministerium fuer Bildung und Forschung, Germany.

**Author details**

1. Institute of Medical Microbiology, Justus-Liebig University Giessen, Schuberstrasse 81, D-35392 Giessen, Germany. “Department of Radiology, Justus-Liebig-University Giessen, Klinikstrasse 33, D-35392, Giessen, Germany.

2. Department of Dermatology, Venereology, and Allergology, Justus-Liebig-University Giessen, Gaffkystrasse 14, D-35392, Giessen, Germany.

**Received:** 22 August 2013 **Accepted:** 27 March 2014

**Published:** 29 March 2014

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Cite this article as: Imirzalioglu et al. Distinct polymicrobial populations in a chronic foot ulcer with implications for diagnostics and anti-infective therapy. *BMC Research Notes* 2014, 7:196.