Temporal Temperature Gel Electrophoresis to Survey Pathogenic Bacterial Communities: The Case of Surgical Site Infections

Romano-Bertrand Sara[1,2], Parer Sylvie[1,2], Lotthé Anne[1,2], Colson Pascal[3]
Albat Bernard[4] and Jumas-Bilak Estelle[1,2]
[1]University Montpellier 1, Equipe pathogènes et environnements, UMR 5119 ECOSYM
[2]University Hospital of Montpellier, hospital hygiene and infection control team
[3]University Hospital of Montpellier, Cardio-thoracic intensive care unit
[4]University Hospital of Montpellier, Cardio-thoracic surgery unit
France

1. Introduction

The main objective of this chapter is to review 16S rRNA gene-based PCR-Temporal Temperature Gel Electrophoresis (TTGE) methods with emphasis on its use in medical microbiology and infectious diseases. As an example of application, we describe optimization, validation and results of an original approach for exploring the microbiology of surgical site infections with a focus on particular constraints related to low microbial load found in this setting. Finally, PCR-TTGE will be situated in the evolution of medical microbiology and infectious disease medicine toward the analysis of complex microbial communities.

2. State of the art

2.1 From human microbiome to pathogenic bacterial communities

At the early 21th century, studies on human-associated bacteria showed that there are at least 10 times as many bacterial cells as human cells, i.e. 10^{14} bacterial cells in the human gut (Turnbaugh et al., 2007). The current estimation of the number of genes in the human genome is about 23000 (Wei & Brent, 2006). Based on the diversity of gut microbes and the average number of genes contained in one bacterial genome, the diversity of bacterial genes in human gut was guessed to be 100 times greater than that of our human genome (Bäckhed et al., 2005). This number seems to be underestimated, since a more recent publication estimates to more than 9.000.000 the number of unique genes in human gut bacterial community (Yang et al., 2009). This huge community is named the human microbiome, a term coined by Joshua Lederberg in 2001 "to signify the ecological community of commensal, symbiotic, and pathogenic microorganisms that literally share our body space" (Lederberg & McCray, 2001). A new concept is to consider human organism as an assemblage of human and bacterial cells organized into organs, tissues, and cellular

communities amounting to a super- or a hetero-organism. Metagenomics show clear differences between microbiomes in various body sites and together with metatranscriptomics and metaproteomics reveal how microbiomes contribute to organ and tissue functions. Consequently, human biology can no longer concern itself only with human cells: microbiomes at different body sites and functional metagenomics must be considered part of systems biology (Pflughoeft & Versalovic, 2011). The biological concept of hetero-organism further evolves into new medical conception on health and disease, involving not only human genes and cells but also related genomes from environment around and inside human body. For instance, the change in gut microbiome is associated with local pathology such as Crohn's disease but also with systemic diseases such as obesity (Turnbaugh et al., 2009), diabetes (Cani et al., 2007) and hypertension (Holmes et al., 2008), among other chronic diseases (Proal et al., 2009; Lampe, 2008). The microbiome of various human body sites has already been described for the digestive tract, the mouth, the skin, the genital tract and it will continue with the « Human Microbiome Project » (Turnbaugh et al., 2007).

Microbiome and its disturbances are now a major area of microbiology research, as attested by the leading publications produced in this field. However, these advances in 'microbiomology' have not yet change the patterns of medical reflection. In the field of infectious diseases, bacteriologists still strive to obtain a pure culture preferentially isolating known pathogens and focus on the morphology, physiology, and genetics of the small subset of the microorganisms in the body that are known to cause disease. Hence, most bacteria present in a community are neglected and so are the complex relationships among members of the community. It is well-known that a large range of endogenous bacteria can cause opportunistic infections such healthcare associated infections (HAI), consecutive to microbiome disequilibrium, and that strict pathogens must cross the cutaneous or mucosal barriers covered with local microbiota before developing their virulence. Therefore, considering the whole human microbiota is required to better understand the physiopathology of infections and improve their prevention and treatment of bacterial infectious diseases that remain major challenges in public health. Confronting microbiomes analyses to clinical issues will warrant these analyses to be good predictors and markers in infectious disease. This implies that microbiomes analyses become routine, which is not yet the case.

2.2 How to explore bacterial communities?

Bacterial communities are classically assessed through culture-dependent methods based on colony isolation on solid medium, sometimes after enrichment by growth in liquid medium. This way, the description of complex communities inevitably involved time-consuming steps of growth on multiple media under varying conditions, while the full description of the community is not insured. Indeed, it is now obvious that the real microbial diversity is poorly represented by the cultured fraction, and conventional culture techniques have been shown to explore less than 1% of the whole bacterial diversity in environment samples, such as soil samples (Riesenfeld et al., 2004). It is also estimated that as much as 20% to 60% of the human-associated microbiome, depending on body site, is unculturable (NIH HMP Working Group et al., 2009). A renewal of studies on complex bacterial communities has been made possible by molecular methods that allow direct analysis of bacteria present in a

sample, while avoiding the bias of cultivability. The emergence of molecular ecology and metagenomics offers the potential of determining microbial diversity in an ecosystem without prior laboratory enrichment, isolation and growth on artificial media thanks to sophisticated methodological and computational tools. Generally, culture-independent approaches allow a precise description of an ecosystem by assessing its genetic diversity. As an example of molecular ecology efficiency, Dekio et al (2005) show that the total skin microflora explored by culture-independent molecular profiling is greater than previously believed, finding 22 potentially novel members, comprising 9 species and 13 phylotypes not yet described as members of skin microbiota (Dekio et al., 2005).

Metagenomics examine the complexity of the community by sequencing genomic libraries made from DNA extracted directly from the sample containing a complex mix of different bacteria. The complete metagenomic approach will give the total gene content of a community, thus providing data about biodiversity but also function and interactions (Tyson et al., 2004). For the purpose of biodiversity studies, metagenomics can focus on one common gene shared by all members of the community. The most commonly used culture-independent method relies on amplification and analysis of the 16S rRNA genes in a microbiome (Nossa et al., 2010).

16S rRNA genes are widely used for documentation of the evolutionary history and taxonomic assignment of individual organisms because they have highly conserved regions for construction of universal primers and highly variable regions for identification of individual species (Woese, 1987). The 16S rRNA gene, in spite of some recognized pitfalls (von Wintzingerode et al., 1997), remains today the most popular marker for studying the specific diversity in a bacterial community. The different 16S rRNA genes representative of the community are amplified by PCR and then separated and identified either by cloning and Sanger sequencing or by direct pyro-sequencing (Nossa et al., 2010). Tools for sequence-specific separation after bulk PCR amplification, such as T-RFLP (Terminal-Restriction Fragment Lenght Polymorphism) (Kitts, 2001), D-HPLC (Denaturing High Performance Liquid Chromatography) (Penny et al., 2010), CDCE (Constant Denaturing Capillary Electrophoresis) (Thompson et al., 2004), SSCP (Single Stand Conformation Polymorphism) (Ege et al., 2011), DGGE (Denaturing Gradient Gel Electrophoresis) (Muyzer et al. 1993), TGGE (Temperature Gradient Gel Electrophoresis) (Zoetendal et al., 1998) and TTGE (Temporal Temperature Gradient Gel Electrophoresis) (Ogier et al., 2002), can also be used. Methods based upon separation in denaturing electrophoresis appear particularly suitable for the routine follow-up of microbiomes with low or medium diversity (Roudière et al., 2007). They provide a "fingerprint" of the community diversity.

2.3 16S rRNA gene PCR-TTGE: Advantages and limitations

PCR-TTGE is a PCR-denaturing gradient gel electrophoresis that allows separation of DNA fragments in a temporal gradient of temperature (Yoshino et al., 1991; Ogier et al., 2002). PCR amplicons of the same size but with different sequences are separated in the gel. In a denaturing acrylamide gel, DNA denatures in discrete regions called melting domains, each of them displaying a sequence specific melting temperature. When the melting temperature (Tm) of the whole amplicon is reached, the DNA is denatured creating branched molecules. This branching reduces DNA mobility in the gel. Therefore, amplicons of the same size but with different nucleotide compositions can be separated based on differences in the

behavior of their melting domains. When DNA is extracted and amplified from a complex community, TTGE lead to the separation of the different amplicons and produce a banding pattern characteristic of the community. The gradient obtained by varying the temperature over time in TTGE generally produces more clear and reproducible profiles than does the chemical gradient in DGGE.

Direct observation and counting bands on the TTGE profile provides a diversity score that roughly corresponds to the number of molecular species in the sample. However, it must be remembered that two amplicons with different sequences can give identical migration distances when their Tm are identical. The banding profile can be further analyzed by the affiliation of each band to a species or other taxon. Affiliation can be realized by comparing the migration distance of each band to a molecular ladder, named diversity ladder (Roudière et al., 2009; Ogier et al., 2002), constructed by using amplicons corresponding to species known to be representative of the community under study. A more accurate analysis of each band can be achieved by cutting bands from the gel, extracting DNA from bands and sequencing. This way the diversity ladder will be completed and updated. A method associating the use of diversity ladder with sequencing has shown its efficiency in describing bacterial communities of low complexity such as the gut microflora of neonates (Roudière et al., 2009). Such an approach is simple enough to survey dynamics of bacterial communities on a wide range of samples, particularly in health and disease (Jacquot et al., 2011).

PCR-TTGE appears not suitable for investigating highly diverse communities. This limit is due to number of bands that can be separated within the length of the gel. Optimization of TTGE conditions allows separation of bands by a minimum of 0.1 mm over all the gel length. We showed that the number of specific bands separable by TTGE could not exceed about 50 for the migration of an artificial diversity ladder. For instance, a ladder contained 53 different bacterial species found in stool of neonates can be efficiently separated in the optimized TTGE conditions (Roudière et al., 2009). For samples obtained from natural ecosystem, TTGE would be difficult to interpret if the diversity exceeds 25 to 30 bands.

Prior to TTGE migration, other technical steps are limitative and should be carefully considered and optimized. Particularly, DNA should be recovered and amplified from all the genotypes in the community, i.e. extraction and PCR should be as universal as possible. Special attention should be given to *Firmicutes* and *Actinobacteria* because they display thick and resistant cell wall. The extraction efficiency should be tested on a wide panel of bacteria to scan a large range of bacterial types. Extraction is generally improved by the use of large-spectrum lytic enzymes and/or by a mechanical grinding (Roudière et al., 2009; Le Bourhis et al., 2007).

The PCR itself is a cause of limitations in the PCR-TTGE approach. Molecular methods are often praised for their sensitivity. However, this detection sensitivity can fail when complex samples are analyzed. For example, detection thresholds of 10^3-10^4 CFU/mL are currently described for universal PCR-TTGE or PCR-DGGE (Le Bourhis et al., 2007; Temmerman et al., 2003; Roudière at al., 2009). The detection limit in PCR-TTGE cannot be easily assessed as it depends on both CFU/g count of each species and the relative representation of species in the community. Minor populations of less than 1% of total population are undetectable by PCR-TTGE. This breakpoint is commonly reported for denaturing-gel-based methods used in microbial ecology (Ogier et al., 2002; Zoetendal et al., 1998; Roudière et al, 2009).

PCR can also be affected by preferential PCR amplification that may hinder the detection of some genotypes when a complex mix of DNA molecules is used as template. Preferential PCR amplification can be caused by primer mismatches at the annealing sites for some genotypes or by a lower rate of primer hybridization to certain templates due to a low local denaturation (Kanagawa, 2003). PCR carried out on complex bulk DNA can produce heteroduplexes particularly in later cycles when primer concentration decreases and the concentration of PCR products is high (Kanagawa, 2003). Chimeric amplicons can also be formed in later PCR cycles when template concentration is high enough to allow the re-annealing of templates before primer extension (von Wintzergerode et al., 1997; Kanagawa, 2003). All these artifacts can generate additional signals that do not correspond to genotypes in the sample. Heteroduplexes and chimera produce additional bands in the TTGE pattern that lead to an overestimation of the diversity. These artifactual bands can be detected either on the basis of their very short migration distance or by sequencing. Consequently, the crude diversity index determined by simple band count should be optimized after exclusion of heteroduplexes and chimeric bands.

Last but not least, a major parameter in community studies is the choice of a molecular marker allowing the genotyping of the whole community. The notion developed by Woese that rRNA genes could identify living organisms by reconstructing phylogenies resulted in the adoption of 16S rRNA gene in microbiology (Woese, 1987). Its universality and the huge number of sequences stored in databases have established 16S rRNA gene as the "gold standard" not only in microbial phylogeny, systematics, and identification but also microbial ecology (Case et al., 2007).

For the purpose of identifying an isolated bacterial strain, the complete 16S rRNA gene (1500 bp) is generally used, giving accurate affiliation to a species in most cases. In PCR-TTGE experiments, the amplified fragments are short (200 to 400 bp) to allow migration in polyacrylamide gel. The fragment amplified should contain hypervariable regions of the 16S rRNA gene in order to compensate for the lack of information due to the small sequence size by a high rate of mutation. Bacterial 16S rRNA genes comprise nine hypervariable regions, V1-V9, exhibiting sequence diversity among species (Van de Peer et al., 1996). In most studies, the V3 region located in the 5' part of the gene is chosen (Jany & Barbier, 2008). However, the phylogenetic information is sometimes insufficient to achieve species identification. Depending on the bacterium, sequences provide identification to the genus or family level only. Consequently, the diversity of the community is not described by a list of bacterial species but by a list of operational taxonomic units (OTUs) corresponding to the lower taxonomic level being accurately identified.

At the genomic level, rRNA genes are generally organized in multigene families (Acinas et al., 2004). The members of a rRNA multigene family are subject to a homogenization process allowing the multiple gene copies to evolve in concert. In a concerted evolution mode, mutation occurring in one copy will be fixed in all of them or lost from all. Thus, rRNA sequences show low variability within species, subspecies or genome (Liao, 2000). However, intra-genomic heterogeneity in the form of nucleotide differences between 16S rRNA gene copies are often described. For examples micro-heterogeneity has been identified in *Escherichia coli*, *Mycobacterium terrae*, *Paenibacillus polymyxa*, members of the classes *Mollicutes*, and *Actinomycetales* (Teyssier et al., 2003). Analyzing of complete genome sequences has recently assessed intra-genomic heterogeneity. For genomes with more than

one rRNA operon, 62% display some degree of sequence divergence between 16S rRNA loci in a same genome (Case et al., 2007). In PCR-TTGE, the intra-genomic 16S rDNA heterogeneity can lead to multiple bands for a single OTU and then to an overestimation of OTU diversity. This pitfall inherent to the 16S rRNA gene marker will be avoided by band sequencing. However, as a pre-requisite of diversity analysis by PCR-TTGE, the major known species expected in a particular ecosystem should be individually studied by TTGE in order to explore heterogeneity in 16S rRNA gene copies (Roudière et al, 2007; Michon et al., 2011).

Alternative markers can also be proposed such as *rpoB* (Case et al., 2007) but universal *rpoB* PCR primers allowing the exploration of the whole bacterial diversity can not be designed (personal data) and the databases remain poor in *rpo* sequences.

Several authors remarked that culture-independent methods regularly fail to identify species obtained using culture-dependent methods (Jany & Barbier, 2008). By contrast, culture-dependent methods have yielded information on the structure of microbial populations but they are limited by the in vitro growth capacity of most bacteria in a community. Such a discrepancy is not observed for all the communities studied. For instance, culture dependant and independent approaches of the premature neonate gut microbiome give globally congruent results (Roudière et al., 2009). However, it is accepted that culture-independent methods remain the only approach for monitoring the rapid dynamics of microbial communities. Nevertheless, the two types of methods reveal different images of the same community and combining culture-dependent and culture-independent methods may be worthwhile to obtain a more accurate view of the structure of the microbial community (Case et al., 2007).

In spite of the limited growth capacity of most bacteria, culture-dependent methods remain the sole approach available for monitoring sub-populations selected on the basis of phenotypic traits such as dependence to metabolites or resistance to antimicrobial agents. In this context, PCR-TTGE can be used after culture in specific conditions in order to describe the diversity of cultivable population. The colonies growing in diverse conditions can be bulked and further analyzed by PCR-TTGE as described before. This culture- and genetic-based mixed approach is particularly suitable to describe dynamics of populations according to their level of resistance to antimicrobial drugs in natural environments (Vanhove et al., 2011).

Culture-independent approaches have previously shown their interest in cardiology to detected new or atypical infectious agents (Marchandin et al., 2009; Daïen et al., 2010). Considering the interest and limitations of 16S rRNA PCR-TTGE, we will proposed a protocol for describing the diversity and following the dynamics of the bacterial community that colonize surgical wound of the patient during hospitalization for cardiac surgery. We will show how 16S rRNA PCR-TTGE is particularly suited to the low bacterial diversity encountered in aseptic surgical settings, where antibiotic prophylaxis and cutaneous antisepsis effectively reduce the bacterial load of patients.

In addition, one example of the use of 16S PCR-TTGE in a culture-dependent analysis will be detailed. This approach associates determination of Minimal Inhibitory Concentration (MIC) at the community level and determination of the diversity by 16S PCR-TTGE in the resistant sub-population at each concentration of antimicrobial agent.

3. An original method of PCR-TTGE to learn more about the physiopathology of surgical site infection in cardiac surgery

3.1 Surgical site infections: State of the art

3.1.1 Epidemiology of surgical site infection

Surgical site infections (SSIs) are among the most frequent healthcare associated infections (HAIs), along with urinary tract and pulmonary infections, and remain an unresolved problem for modern medicine, their occurrence having significant impact on patient morbidity, length of stay and cost of care. Data from longitudinal surveillance studies show SSI rates of 1 to 5% (Klevens et al., 2007; Astagneau et al., 2009; de Lissovoy et al., 2009), whereas higher rates are reported from interventional studies, where control groups can have up to 8.5% infection rates (Bode et al., 2010; Perl et al., 2002), owing to different population case mixes. Large-scale epidemiological studies have identified risk factors for SSI that can be grossly classified as related to patient condition, surgical procedure and environment. The American National Nosocomial Infection Surveillance system (NNIS) developed an easily calculated risk index that combines the patient-related risk assessment of the American Society of Anesthesiologists (ASA) score, and 2 surgical procedure-related factors: type of surgery as defined by pre- or per-operative microbial contamination (Altemeier classification, from I – clean surgery- to IV – septic surgery), and duration of operation exceeding 75th percentile for a given procedure. For all categories of surgery, there is a linear increase in the incidence of SSI when the NNIS risk index increases (Coello et al., 2005). Beside these surveillance-derived risk assessment scores, prospective controlled studies identified many more factors associated with a higher risk of SSI. Most important are poorly controlled diabetes mellitus, malignant diseases, smoking, advanced age, per operative hypothermia, emergency surgery (Coello et al., 2005). Specific risk factors have been identified for cardiac surgery: obesity, pre operative myocardial infarction, chronic obstructive broncho-pulmonary disease, duration of extra corporeal circulation, early post operative bleeding, combined valve and coronary bypass procedures… (Filsoufi et al., 2009).

In the mid 1980s, 30 to 35% of nosocomial (i.e. hospital-acquired) infections were deemed evitable (Haley et al., 1985). A recent analysis of infection control interventional studies estimates that as many as 26 to 54% of SSIs could be avoided by comprehensive implementation of evidence-based prevention strategies, foremost of which are pre operative cutaneous antisepsis, no pre operative shaving of surgical site, timely antibiotic prophylaxis and strict per operative glycemic control (Umsheid et al., 2011).

Still, even when all known preventive measures are implemented, even in low-risk (i.e. clean, non urgent) surgery for low risk patients, SSI can occur. This seemingly irreducible rate of "inevitable" infections raises the problem of how surgical site infections develop. Success of preventive measures based on antisepsis and optimization of patient status compounds the hypothesis that infection results from disequilibrium between host defense mechanisms and microbial infectiveness. In a 10-to-1 inequity between human cells and colonizing microbes, the balance of power in ensured by integrity of skin and mucous membranes, both obviously disrupted by surgery (Wenzel, 2010). However, the intimate mechanisms of infection are not known, starting with the origin of germs involved.

3.1.2 Are surgical site infections related to endogenous or exogenous bacteria?

SSIs are 4.5 times more frequent in patients with nasal carriage of *Staphylococcus aureus* compared to non-carriers. In carrier patients who developed SSI, *S. aureus* isolated from infection site was identical to the one isolated from anterior nares in 84% of cases (Perl et al., 2002). The case for an endogenous bacterial origin is indirectly made by the 60% reduction rate in *S. aureus* SSIs obtained by thorough pre operative decontamination (Bode et al., 2010). Nasal carriage of *S. aureus* was found to be associated with a higher rate of SSIs in orthopedic surgery in a French multicentric study (Berthelot et al., 2010). However, in this study, only 27% of *S. aureus* infections were molecularly linked to an endogenous strain. This can be due to insufficient sensitivity of carriage detection, or to the fact that infections don't necessarily have an endogenous origin.

Indeed, bacteria involved in SSIs can also originate from an exogenous source in the per operative environment. Several studies report cases of cross contamination of surgical sites with bacteria molecularly linked to health care professionals or other patients (Perl et al., 2002). Contamination of surgical instruments and devices increases over time inside the operating room (Dalstrom et al., 2008). Under different air treatment devices, air contamination can vary from 8 to 34% (Knobben et al., 2006). In the air above the surgical site, *S. aureus* and coagulase negative staphylococci can be found, mostly molecularly linked to nasal and pharyngeal carriage by operating team personnel (Edminston et al., 2005).

3.1.3 From contamination to infection

The origin of germs notwithstanding, what induces ordinarily commensal germs to become pathogenic is mostly unexplained. Indeed, in spite of the frequent presence of germs around and in the surgical site (up to 4.1% deep tissue samples found positive in clean orthopedic surgery (Byrne et al., 2007)), only very few infections actually develop.

Antibiotic prophylaxis certainly thwarts the development of most SSIs, at least insofar as bacterial load remains low and antibiotic spectrum and pharmacokinetics are adapted to the germs and tissues involved (Classen et al., 1992). However, the occurrence of SSIs in spite of adequate prophylaxis leads to consider the mechanism of infection from a bacterial point of view. Infections are often the consequences of disequilibrium in the relationships inside the microbiota or between the microbiota and the host. SSIs are typically infections related to microbiota disequilibrium, their major etiologic agents being members of the skin microbiota such as *S. aureus,* coagulase-negative Staphylococci and *Propionibacterium acnes.* The mechanism of selection of these bacteria from the skin community remains unknown and could be highlighted by dynamic survey of the microbial re colonization by a suitable method. Exploring the microbiota disequilibrium and its dynamics should provide insights into the mechanisms involved in SWI infections.

We hereafter present the use of TTGE in culture-independent and culture-based approaches to study the dynamics of bacterial communities involved in SSIs. The study was undertaken in the setting of clean cardiothoracic surgery, because SSI rates remain unacceptably high in coronary artery bypass grafts (CABG), in spite of ongoing efforts to minimize patient- and procedure-related risk factors (Filsoufi et al., 2009).

3.2 Culture-independent approach: Use of TTGE to survey the dynamics of bacterial communities involved in surgical wound colonization and infection

3.2.1 Patients and samples

Forty cotton swabs were collected from 5 patients who underwent CABG surgery at the Service of Thoracic and Cardiovascular Surgery of the Montpellier University Hospital (France). For each patient, swabs were sampled during intervention at the surgical site, superficially and deeply. The first sample was taken on the skin after cutaneous antisepsis just before incision and the second one in sub-cutaneous tissue once incision made. Thirdly, sternum edges were sampled after sawing. The fourth sample was mediastinal tissue after positioning of sternal retractors. At the end of the operation, mediastinum, sternum edges, sub-cutaneous tissue and skin were sampled again.

3.2.2 Cell lysis, extraction of bacterial DNA and PCR amplification

Bacterial genomic DNA was directly extracted from bulk cells present on cotton swabs using an enzymatic method (MasterPure Gram positive DNA purification kit, EPICENTRE Biotechnologies ®) according to the manufacturer's recommendations with modifications as described by (Roudière et al., 2009). This method has been previously described as efficient on a wide range of bacteria including Gram-positive bacteria (Roudière et al., 2009; Jacquot et al., 2011). A fragment about 1465 bp of the 16SrRNA gene was amplified using the primers 27f (5'-GTGCTGCAGAGAGTTTGATCCTGGCTCAG-3') and 1492r (5'-CACGGATCCTACGGGTACCTTGTTACGACTT-3'). The PCRs were carried out in 50 μL of reaction mixture containing 200 nM of each primer (Sigma Genosys), 200 nM each dNTP (Fermentas), 1U of *Taq* polymerase (Promega) in the appropriate reaction buffer, and 1 μL of crude DNA extract as the template. PCR conditions were 30 cycles of 1 min at 94°C, 1 min at 65°C, and 2 min at 72°C.

The 199-bp fragment (from position 338 to position 536, *Escherichia coli* numbering) overlapping the 16S rDNA V2-V3 variable region (Neefs et al., 1993; Sundquist et al., 2007) was amplified using the primers HDA1-GC (primer HDA1 with a fragment rich in GC – the 'GC clamp' – added to the 59 extremity) and HDA2 (Ogier et al., 2002). The reaction mixture (50 μl) consisted of 200 nM of each primer (Sigma Genosys), 200 mM each dNTP (Fermentas), 2.5 U FastStart Taq DNA polymerase (Roche, France) in the appropriate reaction buffer, with 1.8 mM MgCl$_2$. One μL of DNA previously amplified was added to the reaction buffer and the thermal cycling was as follows: 95°C for 2 min; 35 cycles of 95°C for 1 min, 62°C for 30 s, 72°C for 1 min ; and 72°C for 7 min. PCR products were checked by electrophoresis in a 1.5 % agarose gel before TTGE migration.

3.2.3 TTGE migration

TTGE migration was performed in the DCode Universal Mutation Detection System (Bio-Rad Laboratories). Gels were composed of 8 % (w/v) bisacrylamide (37.5: 1), 7 M urea, 40 ml N,N,N9,N9-tetramethylethylenediamine, and 0.1 % (w/v) ammonium persulfate, and were run in 16 Tris/acetate/EDTA buffer at pH 8.3. DNA was loaded on the gel with in-house dye marker (50% sucrose, 0.1% bromophenol blue) using capillary tips. The electrophoresis conditions were 46 V for 16 h with an initial temperature of 63°C and a final temperature of 70°C corresponding to an increase of 0.4°C h^{-1}. In order to obtain thin

discrete bands, a pre-migration for 15 min at 63°C and 20 V was done after loading. Pre-migration and migration were performed with additional magnetic shaking in the electrophoresis chamber. Gels were stained for 15 min with 0.5 mg ethidium bromide ml^{-1} in 1x TAE buffer, washed for 45 min in 1x TAE buffer, and photographed under UV illumination.

3.2.4 TTGE band sequencing

Each TTGE band for further analysis was cut out of the gel with a disposable sterile scalpel to avoid contamination between bands. Gel slices were washed twice in molecular biology grade water and incubated overnight at 37 °C in 10 mM Tris buffer (pH 8.5) to allow DNA diffusion. Amplification of a single 16S rRNA gene V3 region copy was performed using 1 μl band eluate and the primers HDA1 without a GC-clamp and HDA2. The PCR was carried out in 50 ml reaction mixture containing 200 nM of each primer, 200 mM each dNTP, 2.5 mM $MgCl_2$ and 2.5 U Taq DNA polymerase (Promega) in the appropriate buffer. PCR conditions were 94 °C for 2 min; 35 cycles of 45 s at 95 °C, 30 s at 62 °C, 1min at 72 °C; and 10 min at 72 °C. PCR products were checked by electrophoresis in a 1.5 % agarose gel and sequenced on an ABI 3730xl sequencer (Cogenics). Each sequencing chromatograph was visually inspected and corrected. The sequences were analyzed by comparison with Genbank (http://www.ncbi.nlm.nih.gov/) and RDPII databases (http://rdp.cme.msu.edu/) using Basic Local Alignment Search Tool (BLAST) and Seqmatch programs, respectively and affiliated to an OTU as recommended (Drancourt et al., 2000; Stackebrandt & Goebel, 1994).

3.2.5 Optimization of DNA amplification in samples with low load of template

In most assays, the bacterial load is low because samples originated from quasi-sterile sites, i.e. the mediastinum during the surgical intervention. When applied on DNA samples extracted from a low number of bacteria (<10[3]/tube) (Roudière et al., 2009), a single PCR produced a faint or no signal in agarose gel electrophoresis (data not shown). We showed that the signal was equivalent to the positive control for DNA extracted from skin, a site with a resident microbiota but faint for the samples taken from sub-cutaneous and mediastinal tissues, sites expected to be almost sterile (data not shown). To gain in sensitivity, we performed a nested-PCR approach with pre-amplification of the almost complete 16S rRNA gene, further used as template for a second PCR focused on the V2-V3 hypervariable regions of the gene. As expected, nested-PCR resulted in an improved signal in agarose gel for all samples including samples with low bacterial load (Figure 1A and 1B). However, nested-PCR could induce biases such as amplification of contaminant DNA, preferential amplification of a sub-population of template and heteroduplex formation, among others (Park & Crowley, 2010. Consequently, the enhanced quantity of amplified DNA in agarose gel had to be confirmed in TTGE in order to assess the quality of amplified DNA and qualitatively compare single and nested PCR. Figure 2 shows the TTGE profiles after single- and nested-PCR obtained for 4 samples. We observed that nested-PCR led to an enrichment of the profiles while all the bands detected by single PCR-TTGE were also observed in nested PCR-TTGE. The PCR-TTGE profiles presented in Figure 2 displayed neither obvious heteroduplex bands nor smears due to amplification of miscellaneous contaminant DNA. However, preferential amplification was observed particularly for

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sample AD6 In the corresponding TTGE profile, the faint bands seen in single PCR-TTGE profile were very faint in the nested PCR-TTGE profile while original and intense bands appeared in the latter profile. The reproducibility is also satisfactory (data not shown).
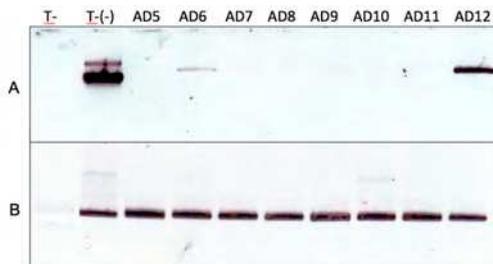

Fig. 1. Migration in agarose gel after single (A) and nested PCR (B) for samples from the patient AD. AD5, skin before incision; AD6, sub-cutaneous tissue after incision; AD7, sternum edges; AD8, mediastinum at the beginning of the operation; AD9, mediastinum at the end of intervention; AD10, edges of sternum before closing; AD11, sub-cutaneous tissue before suture; AD12, skin at the end of the procedure.
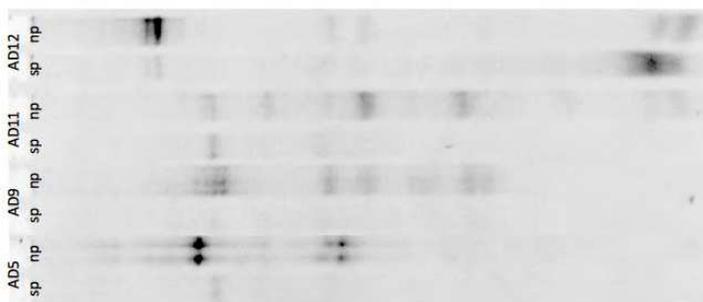

Fig. 2. TTGE migration after single PCR (sp) and nested PCR (np) on samples from the patient AD. AD6 sub-cutaneous tissue after incision; AD9, mediastinum before suture; AD11, sub-cutaneous tissue before suture; AD12, skin at the end of the surgery.

3.2.6 PCR-TTGE profiles and diversity index

About fifteen samples from the sternal region were recovered during hospital stay of each patient undergoing programmed CABG surgery. TTGE profiles were obtained for each sample after nested-PCR and showed clear bands easily separated from each other (data not shown). This result confirmed that 16S rRNA gene PCR-TTGE was a suitable method to survey the bacterial community from skin and wound tissues in cardiac surgery. By numbering the bands in each profile, crude diversities index (DI) were determined. Crude DI varied from 2 to 12 (mean value = 6). The bacterial communities presenting the lower mean value of crude DI (DI=4.2) were sampled on the skin just after preoperative antisepsis. Subcutaneous tissues sampled just before the closure of the wound at the end of the operation displayed the higher crude DI (DI=8.2). The dynamics of evolution of mean crude DI over the surgical operation showed that the bacterial diversity globally increased from the start to the end of the surgery,

from 4.2 to 7.6. The increase of crude DI was also observed at each site of sampling: 4.2 to 7.6 for skin, 7.4 to 8.2 for subcutaneous tissues, 4.4 to 5.2 for sternum banks and 5.4 to 6.8 for mediastin. Figure 3 showed a representative DI dynamics for a patient.
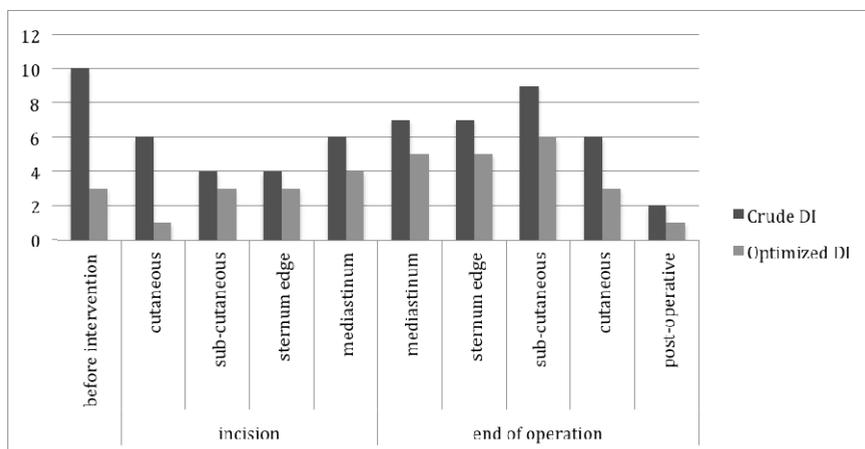

Fig. 3. Representative evolution of crude and optimized DI during the course of the surgical operation of the patient AD.

3.2.7 Operational Taxonomic Units diversity

Each band with specific migration distance was cut from the gel and the eluted DNA was reamplified, sequenced and compared to genetic databases for OTU affiliation. For the five patients presented here, we detected 16 different OTUs (Table 1). Four major OTU, *Staphylococcus* sp., *Flavobacteriaceae*, *Comamonadaceae* and *Propionibacterium* sp., were detected in all patients. *Acinetobacter* sp. and *Corynebacterium* sp. are also frequently detected. In contrast, OTUs such as *Bosea* sp. and *Bacillus* sp. were specific of one single patient. Most major OTUs were present in all patient at all step of the surgery operation and in all sampling sites. *Flavobacteriaceae* and *Comamonadaceae* were atypical Gram-negative bacilli recently described in the skin metagenome (Grice et al., 2009). These atypical Gram-negative bacilli were particularly represented in subcutaneous and in deep tissues (Figure 4). Band identification by sequencing allowed refining the crude DI by the elimination of multiple bands corresponding to a single OTU, as observed for bacteria that displayed 16S rRNA genes heterogeneity. In some cases, the DI dramatically decreased as shown in Figure 3.

3.3 Culture-dependent approach: Use of PCR-TTGE to assess the impact of antimicrobial prophylaxis on the whole skin microbiome

3.3.1 Methods

Sternal skin microbiome was sampled with cotton swabs, before hospitalization, in four patients requiring CABG surgery. This sample reflects the normal resident skin microbiome of the patients. Sternal skin samples were streaked on 5% blood and nutrient agar plates supplemented with different concentrations of cefamandole (0, 0.125, 0.25, 0.5, 2, 4, 8 and 32 µg/mL) and incubated for 24h at 37°C in anaerobiosis and aerobiosis. The resulting colonies

OTU	patient				
	AB	AD	AE	AF	AG
Staphylococcus sp.	X	X	X	X	X
Clostridiaceae				X	
Enterococcus sp.		X			
Acinetobacter sp.		X		X	X
Lactococcus sp.	X				
Streptococcus sp.				X	
Flavobacteriaceae	X	X	X	X	X
Comamonadaceae	X	X	X	X	X
Bosea sp.	X				
Corynebacterium sp.	X	X		X	X
Anaerococcus sp.				X	
Bacillus sp.			X		
Micrococcaceae	X	X	X		
Rhizobiaceae		X			X
Parococcus sp.	X	X		X	X
Propionobacterium sp.	X	X	X	X	X

Table 1. Repartition of the OTUs detected in the skin microbiota of five patients.
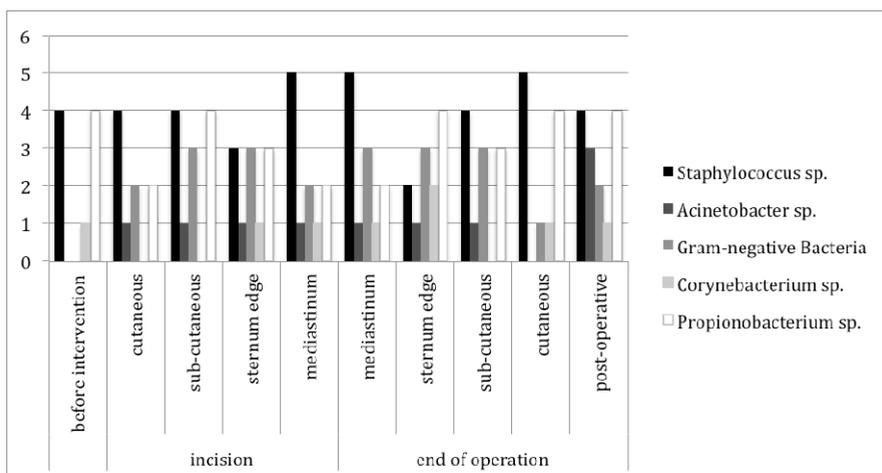

Fig. 4. Number of patients carrying the major OTUs according to the step of the surgery procedure.

were counted and the whole bacterial cultures were then harvested for DNA extraction. DNA extraction, single PCR HDA, TTGE migration and band affiliation to an OTU were performed as described in previous section.

3.3.2 Results: Impact of antimicrobial prophylaxis on skin microbiota

Most samples displayed cultivable microbiota susceptible to cefamandole, i.e. no culture was observed on agar plates with 8mg/L, which was the cefamandole critical concentration used to define susceptibility versus resistance. At lower concentrations (from 0.125 mg/L to 4 mg/L), we observed a decrease of colonies in number and in diversity, comparatively to

the control plate. The mean colony counts went from 80 CFU/plate without antibiotics to 30 CFU/plate with 0.125 mg/L cefamandole and to 6 CFU/plate with 1mg/L cefamandole. All the colonies were bulked and analyzed by 16S rRNA gene PCR-TTGE. Profiles comparison allowed to determine the concentration of antimicrobial agents that inhibited 25%, 50%, 75% and 90% of the microbial diversity and to identify the resistant bacteria to the species level. These data will be related to dynamics of wound re-colonization after surgery.
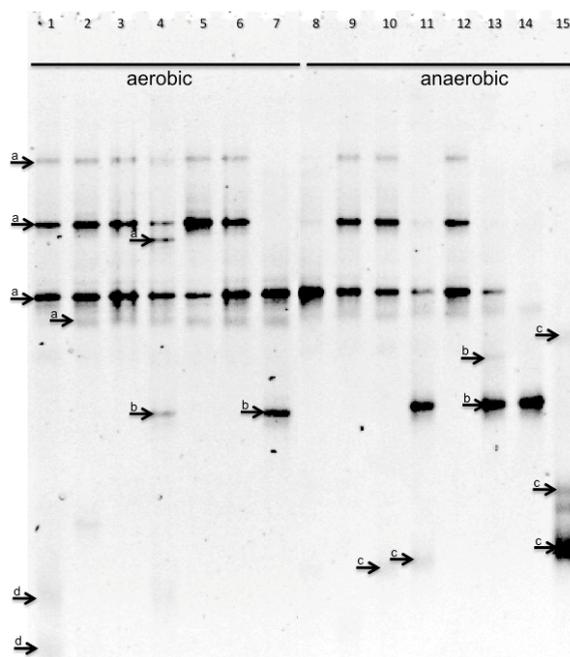

Fig. 5. TTGE gel illustrating the culture-dependent approach on skin sample of the patient CB3. The gel shows differences in banding patterns according to the concentration of cefamandole and the culture atmosphere. Lanes 1 and 8, culture without cefamandole; Lanes 2 and 9, cefamandole 0.125mg/L; Lanes 3 and 10, cefamandole 0.25mg/L; Lanes 4 and 11, cefamandole 0.5mg/L; Lanes 5 and 12, cefamandole 1 mg/L; Lanes 6 and 13, cefamandole 2mg/L; Lanes 7 and 14, cefamandole 4mg/L; Lane 15, cefamandole 8mg/L. Bands corresponding to OTUs are shown with arrow: a for *Staphylococcus* sp., b for *Bacillus* sp., c for *Corynebacterium* sp. and d for *Propionibacterium* sp.

The TTGE profiles from colonies harvested on control plates without antibiotics showed the predominant cultivable population. For the four patients studied herein, the corresponding bacteria belonged to the genera *Staphylococcus* (4 to 6 species) and *Propionibacterium*. For two patients, the cultivable skin microbiota was susceptible to cefamandole because no colonies were observed on plates with more than 8 mg/L, which was the cefamandole critical concentration used to define susceptibility versus resistance. For the two other patients, colonies were observed for 8 mg/L plates incubated in aerobiosis and even for 32 mg/L plates incubated in anaerobiosis. Resistant bacteria belonged to the genera *Bacillus* and *Corynebacterium*. For lower concentrations, which could correspond to the tissue

concentrations in the surgical wound (0.125mg/L to 4mg/L), TTGE profiles showed a modification of the microbiota with loss of bands affiliated to the genus *Staphylococcus* and appearance of other bands affiliated to the genera *Corynebacterium* and *Micrococcus* or to *Bacillus cereus* group. These bands corresponding to OTUs with a decreased susceptibility to cefamandole revealed minor OTUs that were undetected in the total cultivable microbiota, i.e. on plates without antibiotics. For instance in the patient CB (Figure 5), a band corresponding to *Bacillus* sp. was observed from 0,5 à 4mg/L cefamandole and a band corresponding to *Corynebacterium* sp. was observed in at least 8mg/L cefamandole.

4. Conclusion: Why should 'microbiomology' be on the benchtop of the medical microbiologist?

In this experiment we applied PCR TTGE to the study of microbiome dynamics in skin and tissues during a cardiac surgery procedure. We present the results obtained for 5 patients in a culture-independent approach and in 4 patients in a culture-dependent approach. Our results enable us to describe the bacteria involved in surgical site colonization during the procedure. The bacteria detected belonged to OTUs previously described in the skin microbiome (Grice et al., 2009). Gram-positive bacteria belonged to *Bacilli* and *Clostridia* in the phylum *Firmicutes*, and to *Corynebacterium*, *Propionibacterium* and *Micrococcaceae* in the phylum *Actinobacteria*. Gram-negative bacteria mainly belonged to *Proteobacteria* but also to *Flavobacteria.* This result confirmed that the nested-PCR TTGE approach gave a good representation of the skin microbiome even after antisepsis when the bacterial load is very low.

We have showed for the first time that the skin microbiota contaminated the surgical wound at all steps of the surgery in quantities that can be detected by molecular methods. The PCR-TTGE appeared as a suitable method to follow-up the microbiology of a clean wound such as a surgical wound. The approach could also give insights about the activity of antibiotics, e.g. agents used for antibioprophylaxis, at the community level and not only on isolated strains as performed currently.

In order to find relationships between the dynamics of microbiome and the clinical evolution of patients, a cohort of 120 patients will be studied for their surgical wound microbiota and their clinical outcome. Such clinical studies are necessary to understand to physio-pathological mechanism of wound re-colonization and SSI. PCR-TTGE appeared as easy and cost-effective enough to provide microbiological arguments at the community level in large clinical studies. Other very efficient methods such as pyrosequencing are until now not convenient for clinical studies on large cohorts of patients.

More generally the study presented here underlines that is now time for microbiome studies to exit from research labs and to take place on the benchtop of the medical microbiologist.

Current medical microbiology still rests on the shoulders of Leeuwenhoek, Koch and Pasteur. Although every microbiologist knows that a bacterium does not live alone but in complex communities, routine practice still consists in watching a small subset of isolated microbes under microscope and on Petri dishes. It is now clear that the human microbiome has profound effects on health and disease. Ambitious project are in progress such as the international Human Microbiome Consortium and the Human Microbiome Project. High-throughput sequencing and metagenomics allow us to explore new fields of the

relationships between human and bacteria, considering these relationships at the community level. Facing the huge volume of data generated by metagenomics, microbiologists and even more so physicians are perplexed. For instance, Grice et al. admit that "…hairy, moist underarms lie a short distance from smooth dry forearms, but these two niches are likely as ecologically dissimilar as rainforests are to deserts" (Grice et al., 2009). A conceptual revolution is occurring while in medical microbiology practice, skin is still shown as a sheet covered by *S. epidermidis*.

How can we deal with this new world we discover within and around ourselves with our PCR and sequencers? To change our understanding of health and disease, it is now time to develop ways of seeing patterns and interpret them among the staggering biodiversity of microbes. Extending microbial ecology to healthy and diseased microbiota as previously done for mouth (Bik et al., 2010), stomach (Bik et al., 2006), gut (Yang et al., 2009) and skin (Grice et al., 2009) could elucidate the physio-pathology of diseases. For instance, description of the baseline skin microbiome is a step toward testing the therapeutic potential of manipulating the microbiome in skin disorders (Grice et al., 2009). Studies on psoriasis (Gao et al., 2008) describe selective microbial shifts associated with diseases and suggest that therapies might require not only inhibiting the growth of pathogenic bacteria, but also promoting the growth of mutualistic bacteria. Moreover, antibiotic exposure and hygienic practices such as antisepsis modify the skin microbiome. Methods that help understand naturally occurring mutualistic microbial communities will provide insights into the conditions that favor the emergence of antibiotic-resistant organisms.

It is now urgent to confront 'microbiomology' data to clinical data in order to define new indicators for re-evaluating the risk of disease and for adapting therapeutics. Before the generalization of high-throughput metagenomics on the microbiologist's benchtop, alternative methods such as PCR-TTGE or other methods based on denaturant electrophoresis should be considered. Although they are less exhaustive and powerful than pyrosequencing, these approaches are easy to handle in all laboratories and are cost-effective. They can be used to study large cohorts of patients, thus deriving clinical benefits from the conceptual revolution brought by contemporary researches on human microbial ecology.

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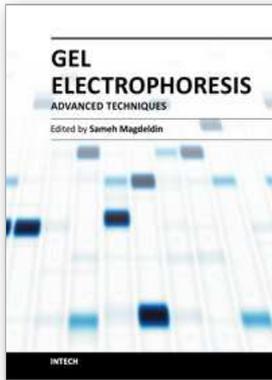
Gel Electrophoresis - Advanced Techniques

Edited by Dr. Sameh Magdeldin

As a basic concept, gel electrophoresis is a biotechnology technique in which macromolecules such as DNA, RNA or protein are fractionated according to their physical properties such as molecular weight or charge. These molecules are forced through a porous gel matrix under electric field enabling uncounted applications and uses. Delivered between your hands, a second book of this Gel electrophoresis series (Gel Electrophoresis- Advanced Techniques) covers a part, but not all, applications of this versatile technique in both medical and life science fields. We try to keep the contents of the book crisp and comprehensive, and hope that it will receive overwhelming interest and deliver benefits and valuable information to the readers.

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