

# The Use of DNA Methods to Characterize Biofilm Infection

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**Abstract** Because of biofilm’s fundamental properties—its polymicrobial nature (genetic diversity) and “viable but not culturable” microbial constituents—clinical cultures are wholly unsuited for evaluating chronic infections associated with biofilm. DNA-based technologies (molecular methods) have a number of advantages for evaluating human infections. Real-time PCR and sequencing technologies are particularly robust for identifying microorganisms in human environments because of development of their methods by the human microbiome project. DNA methods enjoy much higher sensitivity and specificity than cultivation methods for identifying microorganisms regardless of their phenotype. Moreover, real-time PCR can be quantitative in an absolute sense, while sequencing methods yield accurate relative quantification of all constituents of the sampled infection. All methods for microbial identification have biases, yet molecular methods suffer the least from these biases. Although DNA-based identification of microorganisms has the limitation that sensitivities to antibiotics cannot be determined in a Petri dish and must be determined by identifying mobile genetic resistance elements within the microbes, molecular methods are a significant improvement in the identification of microorganisms for human infections and are currently the only reliable technology for diagnosing biofilm infection.

## 1 DNA-Based Testing

*In the land of the blind, the one-eyed man is king—Erasmus.*

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The human microbiome project (HMP) has forever changed how microorganisms will be identified (Chain et al. 2009). The HMP was established to identify and to quantitate bacteria living in normal human environments such as the gut, oral cavity, skin, urogenital, etc. Several challenges for the project were that the microbes in these host environments are polymicrobial, they are not quantifiable by cultivation methods, and they generally exist in a biofilm phenotype. In fact, the vast majority of the species known to inhabit normal host environments are not routinely culturable (Petrosino et al. 2009), which is characteristic of the biofilm phenotype (Fux et al. 2005). These facts led investigators to employ molecular methods.

Molecular methods are based on the idea of direct examination of the bacterial DNA existing in the sample to allow for identification of the bacteria that are present. There has been a very rapid and fluid progression of molecular technologies that can analyze microbial DNA. However, to get any of these molecular technologies to give a meaningful analysis, high-quality DNA first must be obtained. Therefore, one of the most important obstacles to using molecular methods for identifying and quantitating microorganisms in human infections is obtaining good microbial DNA from the sample (i.e., the process of DNA extraction). There are a number of excellent kits and laboratory methods for obtaining microbial DNA from mixed samples (samples that contain both microbial and human DNA). However, each method has different extraction efficiencies, and these efficiencies may vary for the different species within the sample. Yet even with these challenges, many extraction methods can approach 96 % efficiency (Fitzpatrick et al. 2010).

The process of DNA extraction, especially from samples that contain some of the host products, also can extract substances that inhibit later analysis of the microbial DNA. For example, polymerase chain reaction (PCR) is a common method used to amplify microbial DNA, yet the process can be inhibited by substances found in the sample. These PCR inhibitors include complex polysaccharides, bile salts, hemoglobin degradation products, polyphenolic compounds, heavy metals, and, most frequently, large amounts of human DNA (Stauffer et al. 2008). Many of the more common PCR inhibitors can be effectively mitigated, but if the inhibitors cannot be identified and controlled, resampling may be necessary. Once good DNA is obtained from the sample, most current molecular instrumentation can obtain reliable clinical results.

PCR is a widely used method of processing DNA that has a relatively long history of use in the clinic (Krishna and Cunnion 2012; Reddington et al. 2013). PCR utilizes primers that attach to complementary regions of bases in the microbial DNA and, through a polymerase reaction, create copies of this area. This copying process doubles the amount of target sequence with every cycle of the PCR. Real-time PCR has the ability to quantitate, in an absolute sense, how much microbial DNA is in the original sample. The number of cycles required before the real-time signal reaches a detection threshold (cycle threshold number or *ct* number) can be correlated to an absolute number of microbes present in the original sample. This is an extremely powerful feature of real-time PCR that can be used to quantify “bacterial

load” (Verhoeven et al. 2012). However, PCR has several important limitations. Most limiting is the fact that real-time PCR requires a primer sequence to be developed for each species of microorganism present in the sample. With the literally thousands of different microorganisms that can be in human chronic infections, constructing thousands of primers for each analysis is inefficient, costly, and currently not feasible.

There also are a number of different parameters involved in the process of performing PCR, such as chemistries (e.g., Syber Green, TaqMan), platforms (e.g., Roche v. Abbott), factors in plate preparation, etc., that can impact results. Incomplete optimization of these parameters can lead to amplification inefficiencies, inconsistent reproducibility, random PCR products, and other problems. The optimization of chemistries, primers, and instrument variables is focused on improving sensitivity of the primer to the target microbe without sacrificing specificity for the organism (prevention of cross-reactivity with other species). Optimization must also take into account dynamic range so that minor species are detected and quantitated as accurately as the dominant species in the sample.

Diagnostic laboratories painstakingly optimize all of the PCR variables by choosing appropriate instruments, chemistries, and primers to mitigate the potential negative impacts of these variables. However, there are still limits to quantitative PCR methods. For example, even though the reported results will be extremely specific for the microbial species present, due to DNA extraction efficiencies for different species, different amplification efficiencies for different species, and other variables, quantification of the microbes in the sample remains mildly inconsistent. Calculating bacterial load by real-time PCR often yields up to an order of magnitude variation for known quantities (usually lower), yet this seems to be an acceptable level of variability for clinical decision-making.

Although real-time PCR can rapidly yield usable information on bacterial load and identify a limited number of microbial species, it is impractical for PCR to be used alone for the identification and quantification of microbes in most human infections. Investigators in the HMP encountered the same limitations and quickly turned to sequencing (Aagaard et al. 2012). One of the technologies used early on in the HMP was a whole metagenome survey of the microbes present. This methodology looks at all genes present in a sample, which is an excellent way to determine species of fungi, bacteria, and even viruses present in an infection. The problem with determining all the genes present was that it required a massive number of sequencing base pairs (bp-ATCG) for a sample, which only allowed a small number of samples to be evaluated per sequencing run. These surveys also lost some of their quantitative ability (Fodor et al. 2012).

An alternative methodology was developed in which a very specific gene, the ribosomal 16S rDNA gene for bacteria or 18S rDNA gene for fungi, can be amplified through a PCR step and then sequenced. The use of the 16S rDNA gene as an indicator of bacterial taxonomic relationships traces back to the pioneering work of Woese and Fox (1977). This method provides two important pieces of information. Once the 16S or 18S region has been sequenced, it can be compared to a database of known sequences, thus yielding the genus and species

with a high level of confidence. In addition, this method can allow for relative quantification of the microbes present within each sample. The number of “copies” of the gene for each species in the sample can be totaled, allowing for each species to be expressed as a percent of that total number. Although it does not provide for absolute quantification, this method does allow investigators to determine the dominant, major, and minor species within a sample (Rhoads et al. 2012a). Because this approach focused on sequencing only a single gene from each microbe, it allowed for several hundred samples to be analyzed on the same plate in a single run, greatly reducing the cost and increasing the speed of analysis. It was mainly through the development of sequencing technologies and methods that allowed investigators to elucidate fully the microorganisms present in the human microbiome (Morgan et al. 2013).

Sequencing is the molecular method for determining the exact order of nucleotides (i.e., adenine, thymine, cytosine, guanine) of a specific fragment of DNA or an entire genome. Sequencing instruments, such as the Roche 454, the PacBio (Pacific Biosciences), and Ion Torrent (Life Technologies), use different methods, but they all accurately determine the sequences of long segments of specific regions of microbial DNA, such as the 16S rDNA gene for bacteria and the 18S rDNA gene for fungi. These technologies can give a 99 % accurate code for the targeted gene, which is easily translated into taxonomic identification.

The microbial gene that codes for the 16S ribosomal subunit is conserved in all prokaryotic organisms except for a small subgroup of Archaea. The 16S ribosomal DNA has about 1,500 nucleotides, which contain nine hypervariable regions (v1–v9), and allows for the ability to identify bacteria at the species level. Fortunately, v1 can differentiate *Staphylococcus* to a genus level, and if the first three regions (v1–v3) can be sequenced, then the majority of other bacteria can be resolved to a genus level with a high degree of certainty. The 16S ribosomal DNA has been called the genomic fingerprint, and a 400+ nucleotide sequence of the 16S ribosomal DNA region is capable of reliably reading this genomic fingerprint.

Often, sequencing is carried out at multiple points along the 16S gene. It has been demonstrated that sequencing two fragments of the 16S gene consisting of 762 based pairs and 598 base pairs is more accurate in identifying bacteria than a single fragment of 1,343 base pairs (Jenkins et al. 2012). Therefore, sequencing methods often use primer sets consisting of two or more primers that cover different regions of the 16S gene. These primer sets can have some bias in how efficiently they sequence specific bacterial species.

Once sequencing has been completed, a data analysis pipeline is needed to begin processing the data. The data analysis process consists of two major stages: quality checking and diversity analysis. During the quality checking stage, denoising (Quince et al. 2009, 2011) and chimera checking (Haas et al. 2011) are performed on all the reads within the data. Each read is quality scanned and deficient reads are removed from the sample. The primary output of this stage is high-quality sequences. During the diversity analysis, sequences from each sample are run through an algorithm (typically involving a match to a database of known

sequences) to determine the taxonomic information for each sequence. Reference databases exist for sequences from the 16S, 18S, 23S, ITS, and/or SSU regions.

Bioinformatics, the post-analysis processing of the massive data, therefore becomes the overseer of the quality of the reported results to the clinician. It is very difficult for clinicians to abandon the visible, tangible, and familiar microorganism growing in a Petri dish for the very complex “black box” type of results produced by bioinformatics. However, current laboratory regulations requiring strict validation and reproducibility coupled with proficiency testing of unknown samples can allow the clinician to feel very comfortable with these new molecular methods. Also, a closer examination of clinical cultures demonstrates that clinicians may have placed their faith in an insufficient method all along.

## 2 Clinical Cultures: The Land of the Blind

Medical microbiology has clung to cultivation methods even while environmental microbiology migrated to DNA methods for microbial identification decades ago. This failure to take advantage of new technologies to improve microbial identification has left clinicians “blind” to the microbial reality of most infections. Many deficiencies in traditional cultivation methods make routine clinical cultures unacceptable for medical microbiology.

Only a handful of media, such as tryptic soy agar, blood agar, nutrient agar, brain–heart infusion agar, and a few others are used to plate routine samples and they are grown at only one temperature (usually 37 °C) for 24–48 h. These experimental conditions have been worked out to be adequate for *Staphylococcus* species, *Streptococcus* species, *Pseudomonas aeruginosa*, and several other bacteria that can grow under these limitations. However, the vast majority of bacterial and fungal species do not grow under these laboratory conditions. Therefore, hundreds to even thousands of specialty media have been developed along with various algorithms for microbes that require different atmospheres, nutrients, length of time, temperature, etc., to be grown. No other single fact could be more convincing for making the argument that routine clinical cultures are inadequate for diagnosing human infection.

Also, bacteria in the biofilm phenotype are notoriously difficult to grow in routine clinical cultures because they are “viable but not culturable.” Biofilm infections also tend to be polymicrobial. Early investigators at the time of Koch found, “No matter how ingenious the machinery, how careful the researchers, they kept ending up with beakers of mixed bacteria. The inability to get anything but mixed cultures led many scientists to believe that the bacteria had to be in mixed groups in order to thrive, that they could never be separated. . .” (Hager 2006). To solve this problem, Koch developed the methodology of pure culture very similar to that of our current clinical culture.

Koch found on the semiliquid surface of agar infused with necessary nutrients that only one species of bacteria in his clinical sample would propagate and the rest

of the bacteria, “the contaminants,” would not grow or would be outcompeted. What we now know is that the experimental design of the nutrient-enriched agar plate encourages planktonic phenotype propagation of the bacterial species in an exponential growth phase pattern. We also know that the experimental design has significant bias for the bacterial species that propagate well under the experimental conditions of temperature, nutrient, time, etc. This creates a huge selection bias to grow the microorganisms which the medical microbiologists have decided in advance are the pathogens. With molecular methods, we have discovered even more shortcomings of clinical cultures.

Many clinicians continue to hold Koch’s view of one microorganism producing one clinical infection. While this generally may be true for acute infections that are commonly produced by bacteria in the planktonic phenotype, it does not hold true for biofilm infection. Chronic infections are associated with biofilm phenotype bacteria (Del Pozo and Patel 2007) and are often polymicrobial, which confounds the methods of clinical cultures. When molecular methods are compared with clinical culture to identify the microbes, we start to understand why clinical cultures provide little help in managing most chronic infections.

In pleural effusion samples, which tend to be culture negative even when the patient shows clear signs of infection, the use of universal 16S PCR, “bacterial load,” demonstrated bacteria in 82 % of the clinically infected samples, whereas clinical cultures grew bacteria only 55 % of the time. Utilizing a single molecular test improved bacterial identification by 27 %. It should also be noted that this individual PCR test had only 0.9 % false positives whereas clinical cultures had a 2.6 % false positive rate (Insa et al. 2012).

Also, it has been found to be more advantageous to first identify the microorganisms utilizing molecular methods and then select media and growth conditions to cultivate the microorganisms present. Up to 20 different growth conditions were necessary to cultivate microorganisms in a single cystic fibrosis study (Sibley et al. 2011). This demonstrates that the “one size fits all” routine clinical culture is inadequate to handle the diversity of chronic infections.

A retrospective study that evaluated 168 chronic wounds with both clinical culture and molecular diagnostics (PCR and pyrosequencing) revealed the comprehensiveness of molecular methods (Rhoads et al. 2012a). Evaluating chronic wounds at a genus level for bacterial taxa only, cultures identified 17 different bacterial genera, whereas the DNA methods identified 338 bacterial taxa. Cultures underreported the diversity of the wound microbiota, but even more importantly, they failed to identify the most abundant bacteria in the wound over half the time (Rhoads et al. 2012b). Cultures obtained from polymicrobial biofilm infections fail to identify the diversity by a factor of 20-fold and fail to identify the cornerstone genus over half the time.

To improve on the design of the previous study, a prospective study was conducted in which 51 consecutive chronic wounds had a single sample taken from their surface (Rhoads et al. 2012b). The sample was homogenized and a portion was sent for clinical culture, a portion sent for PCR and pyrosequencing, and the remaining saved for further analysis if necessary. Once the clinical culture

was complete and all the sub-plates identified by phenotypic methods (biochemistries) the sub-plates were submitted for sequencing. The results showed that 5 wounds (10 %) were culture negative and 9 of the 46 remaining wounds (19 %) had discrepant results between the bacterial isolate identified by culture versus sequencing. For example, culturing methods identified *P. aeruginosa*, whereas sequencing evaluating the same sub-plate identified *Salmonella enterica*. Once again, culture failed to demonstrate the most abundant species over 50 % of the time (Rhoads et al. 2012a). It may be that one main reason clinicians struggle to manage chronic infections is because traditional culturing methods consistently report minor constituents of the infections rather than the dominant culprits.

Over 68 % of patients receive at least one course of antibiotics for the management of their chronic wounds (Howell-Jones et al. 2005). Unfortunately, multiple studies have demonstrated that treating wounds based on culture results does not improve the outcomes of the healing of the wound (Lipsky et al. 2004, 2011; Siami et al. 2001). This information has led some investigators to conclude that even though pathogens such as *P. aeruginosa* may be present in the wound, the pathogen is not doing any harm. That conclusion is made because when chronic infections are treated with anti-pseudomonal antibiotics specifically for *P. aeruginosa* identified by culture, there is no improvement in wound healing outcomes (Joseph 2013). The confusing results from clinical culture, which leads clinicians and scientists alike to conclude that pathogens may not behave pathogenically or that bacteria don't matter in certain chronic infections (O'Meara et al. 2010), may be due to the inadequacies of the cultivation methods.

Although routine clinical cultures are inadequate for evaluating chronic infections, we must first determine if the proposed replacement (i.e., molecular methods) is any better. That is, will adopting molecular methods improve clinical outcomes for chronic infections produced by biofilm phenotype microorganisms? After all, by growing bacteria, medical microbiologists can apply antibiotic discs and determine the "real-world" sensitivity of the isolated bacteria. Also, even though it has been demonstrated that DNA degrades quite quickly (2–3 days) once the bacteria dies within the host infection (Post et al. 1996), there is no clear determination that the microbial DNA identified by molecular methods is associated with a living bacterial cell. However, in a chronic wound infection model, when wound biofilm was comprehensively diagnosed utilizing molecular methods and the microorganisms identified specifically treated, healing outcomes did improve (Dowd et al. 2011). Regardless, the primary tenant of medicine is for the clinician to fully diagnose the disease, and as demonstrated above, clinical cultures are mostly blind to the microbial reality of polymicrobial biofilm infection.



### 3 Advantages of DNA Diagnosis: The One Eye

An Oslerian (Sir William Osler) model of medicine mandates that the clinician diagnose a malady as fully as possible to formulate the most appropriate treatment available. Evidence-based medicine often requires not only diagnosis before the treatment regimen but also frequent intervals of reevaluation during the treatment to show efficacy. So, no matter the generation of the clinician or which model of medicine to which the clinician ascribes, diagnosis of the condition is fundamental. Diagnosis prior to treatment is especially important in the management of chronic infections.

However, most clinicians treating chronic infections have abandoned the fundamental principle of initial diagnosis. The problem seems to lie not in the clinicians but in the diagnostic tools available. Many different culturing methods have been tried, yet they do not improve outcomes in the treatment of chronic infections. The inadequacy of cultivation methods has led to a de facto management of chronic infections by an educated guess, trial and error method.

The transition toward adopting molecular methods for medical microbiology need not be difficult. For virology there are no other reliable methods other than nucleic acid-based analysis. Almost a decade ago it was established that not only was DNA-based testing more accurate and reliable than clinical culture, but it also had the advantage of reduced time to diagnosis and high throughput (Mothershed and Whitney 2006). New methods have also been developed to identify various different antibiotic resistance determinants while at the same time providing genetic surveillance for new and existing pathogens (Weile and Knabbe 2009). Indeed from 2001 to 2007, 215 novel bacterial species were identified in human infections by sequencing methods with 100 of these new species identified in four or more individual patients (Woo et al. 2008). Molecular methods offer faster and higher throughputs while staying true to the original purpose of identifying and quantifying microbes. Recent studies demonstrate that close to 100 % sensitivity and specificity can be achieved for evaluating clinical infections (Hansen et al. 2010). One issue is that molecular methods may be identifying too many microorganisms, leading the clinician to over treat a specific infection.

DGGE and imaging methods showed that there was much more diversity present in wounds than clinical cultures were reporting (Davies et al. 2004; James et al. 2008). Clinicians managing other chronic infections such as chronic rhinosinusitis (Stephenson et al. 2010), cystic fibrosis (Goddard et al. 2012), middle ear infections (Laufer et al. 2011), and burns utilized molecular methods to show similar findings. It has been generally agreed that these and other chronic infections are associated with bacteria propagating in biofilm phenotype (Del Pozo and Patel 2007). Although molecular methods can identify microbes regardless of their mode of growth, the same is not true for clinical cultures. Molecular technology provides the clinician a more robust understanding of the infection, but also forces the clinician to consider multiple microbial species. At the same time, molecular



methods do not provide any clear information on which species are producing the infection and which species are merely contaminants.

New methods are rapidly developing where microRNA (Martens-Uzunova et al. 2013) and messenger RNA (Mutz et al. 2013) can be sequenced and identified. This will provide critical information as to the inner workings of microbial cells which should provide insight as to strategies being used to cause infection. This may shed light on which microorganisms within the community are behaving as pathogens.

Before a bacterial species can be deemed a pathogen, or more importantly before that species can be dismissed as a contaminant, the clinician must take into account the synergies which arise within a polymicrobial infection. By including multiple bacterial and/or fungal species into a single community, the biofilm achieves numerous advantages such as passive resistance (Elias and Banin 2012), metabolic cooperation (Fischbach and Sonnenburg 2011), by-product influence (Elias and Banin 2012), quorum-sensing systems, an enlarged gene pool with more efficient DNA sharing (Madsen et al. 2012), and many other synergies that give the polymicrobial infection a competitive advantage. It is best to view a biofilm as a single entity possessing multiple genetic resources to allow it to adapt and thrive regardless of the stresses it encounters. In general, a more diverse population (i.e., greater the gene pool) will make the biofilm more robust in terms of its survivability (Tuttle et al. 2011).

Metabolic cross feeding has been well established between genetically distinct species. It has been shown that *Streptococcus gordonii* produces peroxide that can cause *Aggregatibacter actinomycetemcomitans* (Aa) to produce a factor H binding protein which limits the host's ability to kill Aa through a complement mediated lysis (Ramsey et al. 2011). This metabolic cooperation has been identified in numerous polymicrobial models (Dalton et al. 2011; Mikx and van der Hoeven 1975; Kuboniwa et al. 2006).

Waste products, molecules that bacteria produce that are end products and are of no benefit to the metabolizing member, are released into the local biofilm environment. Many of these metabolites such as ammonia, lactic acid, and carbon dioxide can have significant influence on the surrounding microorganisms (Elias and Banin 2012). Studies have demonstrated that *Fusobacterium nucleatum* and *Prevotella intermedia* generate ammonia which raises the pH suitable for *Porphyromonas gingivalis* (Takahashi 2003) and that *F. nucleatum* also provides an increased carbon dioxide environment which increases the pathogenicity of *P. gingivalis* (Diaz et al. 2002).

Passive resistance is when one of the members in the biofilm possesses a resistance factor that can protect other members of the biofilm which do not have the factor. There are numerous biofilm defenses which limit the effectiveness of antibiotics. For example, a beta-lactamase producing strain of *Haemophilus influenza* was cocultured with *Streptococcus pneumoniae* deficient in any resistance factors. *Haemophilus influenza* increased the MIC/MBC of *S. pneumoniae* by amoxicillin (Weimer et al. 2011).

The clinical concern relative to the synergies of polymicrobial biofilm is that the infection will be more severe and recalcitrant to treatment. There are many examples which show that this is indeed the case. Low levels of *P. aeruginosa* mixed with *Staphylococcus aureus* increased infection rates in a rat model (Hendricks et al. 2001). In the mouse model, *Prevotella* increases the pathogenicity of *S. aureus* (Mikamo et al. 1998). *Escherichia coli* produced marked increase in the size of abscess formation with *Bacteroides fragilis* in a diabetic mouse model (Mastropalo et al. 2005). There also is clinical evidence to suggest that polymicrobial infections are more severe (Tuttle et al. 2011).

The synergies and general recalcitrance produced by polymicrobial infections argue for the full evaluation of every infection. This means not only identification of all species present but also their quantification. However, there is currently not enough information to give clear direction on which microorganisms are important to treat. Also, therapeutic tools for managing polymicrobial infections in conjunction with or separate from antibiotics are generally not available. If a clinician has no specific tools to address all the diversity of a polymicrobial infection then is it valuable to get the test in the first place?

## 4 The Clinical Use of Molecular Methods: Two Eyes

Identifying and quantitating the microorganisms present in an infection are only part of the diagnosis of an infection. Clinical findings play the major role in determining if the microorganisms present are harming the host. It is only through stereoscopic vision of laboratory results and clinical observation that we can clearly see the power of the detailed information provided by molecular methods. Just as when sophisticated imaging technologies emerged such as MRI, the full meaning and nuances of the images provided could not be appreciated until there was clinical application and experience.

Clinicians seem to be divided by the information provided by DNA-based testing. The unfamiliar microbes can both elucidate and complicate the diagnosis of chronic infections produced by biofilm. Through years of use of molecular methods in real-world chronic infections (mainly chronic wounds) several important principles have emerged. Uncommon bugs occur commonly in chronic wounds and many chronic infections. The clinical challenge of treating rare microbes is more difficult but doable. Literature searches usually will yield usable treatment options for the genera that are identified. Even though we like to know the species identification, most antibiotics, biocides, quorum-sensing inhibitors, and ancillary treatments work at the genus or even the family level for many microbes. That is, a treatment that would kill a rat would in general kill a mouse. Therefore, unfamiliar microbes for treatment purposes can be grouped with closely related microbes which are more familiar (e.g., *Raoultella planticola* and *Klebsiella* spp.) or categorized by common groupings such as gram negative, gram positive, anaerobic, etc. But all of the grouping and comparing of microbes to form a treatment plan

highlight the main inadequacy of molecular methods, which is the lack of antibiotic sensitivity data similar to that provided by culture methods.

There are several strategies for managing chronic biofilm infections with the lack of antibiotic sensitivity information. First, if the infection is accessible to topical treatment, high concentrations of antibiotics far in excess of resistance factors can overwhelm most mobile genetic element-induced antibiotic resistance. Second, if systemic antibiotics will be necessary then certain mobile genetic elements with limited diversity, such as *mecA* cassettes, *van* genes, and others can be identified by real-time PCR. Third, if sensitivity data is still critical, then molecular diagnosis is still very often the quickest and most cost-effective way to proceed because many microbes are not initially grown in routine clinical culture. By first identifying the microbes of interest by molecular methods, custom nutrients and methods can be used to cultivate microbes for sensitivity work or genomic study (Sibley et al. 2011). With the emerging massive increase in capacity per run, advances in bioinformatics and computing, along with steady decreases in costs, it is becoming feasible to evaluate all the genes in a sample which may allow molecular methods to eventually assess resistance directly in the near future.

Dealing with diversity is made easier by the data provided by DNA-based diagnostics, but caveats remain. Sequencing provides a relative abundance for each species identified in the sample; however, it yields no “absolute” quantification for how much microbial material is present. Real-time PCR has the ability to give reproducible estimates of the number of microbes per gram of tissue (such as  $10^5/\text{g}$ ) which is termed the “microbial load” or “bacterial load.” Several factors can fictitiously lower the value for “microbial load,” such as inefficient extraction, decreased primer efficiency, and small variations throughout the analysis. As a result, a low “microbial load” should never be discounted as “not a significant infection.” The diagnosis of infection is a clinical decision; therefore, chronic infection itself should always dictate treatment. To evaluate the progression or improvement of an infection it may be necessary to have the lab run the initial sample with subsequent samples in the same run to mitigate these variations, which allows for better comparison.

Quantification of microbes in the polymicrobial infections often encountered in biofilm infection is indispensable. For example, if a sample contains just 1 % MRSA but the bacterial load is  $10^8/\text{g}$  then there are still  $10^6$  MRSA even though it is a minor component of the biofilm. So MRSA coverage would be reasonable. But 1 % MRSA with a bacterial load of  $10^5/\text{g}$  ( $10^3$  MRSA) requires only observation which can greatly reduce the use of first-line MRSA antibiotics.

The diversity can be daunting at first, but it is amazing how the many disparate microbes resolve down to treatment groups that require only one or two treating agents. For example, a group of microbes in chronic wounds consisting of MRSA, *Streptococcus*, *Peptoniphilus*, *Anaerococcus*, *Bacteroides*, *Pseudomonas*, and *Serratia* can effectively be treated with the use of clindamycin and amikacin. By collapsing the gram positives and anaerobes into one treatment group covered by clindamycin and then covering the gram negatives with amikacin, only two

antibiotics are needed. In fact, high-dose (250 times MIC) amikacin can also provide double coverage for MRSA.

One study showed that by just adding the ability to assess chronic wounds with molecular methods (PCR and sequencing), the use of expensive first-line methicillin-resistant *S. aureus* (MRSA) treatments was greatly reduced (Wolcott et al. 2010). Molecular methods identified *S. aureus* along with the *mecA* cassette in a majority of the wounds evaluated, yet the quantification showed that MRSA was a minor population (less than 1 % of the bacteria present) and therefore was observed and not actively targeted by antibiotic therapy. Wound care outcomes were improved over standard of care with molecular diagnostics used in this manner. The study demonstrates that using currently available treatments directed by a better understanding of the microbial diversity in question improves outcomes.

Now that molecular tools are available to fully define an infection, it will be up to clinicians to develop appropriate solutions. For example, in the companion study to the one noted above, personalized gels to address what were considered the important species identified within the wound biofilm (usually greater than 1 %) were developed to treat each patient. Molecular diagnostics along with multivalent personalized treatment yielded much better healing outcomes (Dowd et al. 2011).

## 5 Conclusions

Dealing with the complexity of the results is just the beginning—DNA diagnostics face other barriers in routine clinical use. Clinicians must deal with accessibility, choosing the appropriate laboratory for the analysis, and, as always, cost. Yet the cost of DNA extraction, sequencing, bioinformatics, etc., currently rivals cultivation methods and will continue to drop rapidly. Accessibility is still a barrier.

Technologies now exist which very easily could move molecular diagnosis to the bedside in the next several years. Until then, reference laboratories currently offer the best choice of different DNA diagnostic tests utilizing multiple platforms. Nevertheless, the main barrier for general acceptance is the level of enthusiasm of the clinician for translating this technology into managing infections in individual patients. Not until clinicians embrace molecular methods for identifying and quantitating microbes will molecular methods revolutionize the management of chronic infections.

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