

New methods for the detection of orthopedic and other biofilm infections

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Abstract

The detection and identification of bacteria present in natural and industrial ecosystems is now entirely based on molecular systems that detect microbial RNA or DNA. Culture methods were abandoned, in the 1980s, because direct observations showed that < 1% of the bacteria in these systems grew on laboratory media. Culture methods comprise the backbone of the Food and Drug Administration-approved diagnostic systems used in hospital laboratories, with some molecular methods being approved for the detection of specific pathogens that are difficult to grow *in vitro*. In several medical specialties, the reaction to negative cultures in cases in which overt signs of infection clearly exist has produced a spreading skepticism concerning the sensitivity and accuracy of traditional culture methods. We summarize evidence from the field of orthopedic surgery, and from other medical specialties, that support the contention that culture techniques are especially insensitive and inaccurate in the detection of chronic biofilm infections. We examine the plethora of molecular techniques that could replace cultures in the diagnosis of bacterial diseases, and we identify the new Ibis technique that is based on base ratios (not base sequences), as the molecular system most likely to fulfill the requirements of routine diagnosis in orthopedic surgery.

Background

Biofilm infections were defined by Costerton *et al.* (1999), in a review in science, and were seen to encompass all device-related infections and a significant proportion of other chronic bacterial diseases. The characterization of an infection as being a biofilm infection is universally based on the unequivocal demonstration, by direct microscopy, of matrix-enclosed microbial communities within or upon the affected tissues or prostheses (Stoodley *et al.*, 2002). Biofilm infections have increasingly come into prominence, in the past three decades, because acute bacterial diseases that are caused by planktonic bacterial cells have been largely controlled by the development of specific vaccines and broad-spectrum antibiotics (Costerton, 2007). The clinical characteristics of biofilm infections are manifestations of the

mode of growth of the causative organisms, in that their altered phenotype makes them resistant to most known antibiotics (Nickel *et al.*, 1985), and in that their protective matrices make them resistant to host defenses. Chronic diseases (e.g. tuberculosis) are added to the burgeoning list of biofilm infections almost monthly, as direct microscopy shows that the causative organisms (e.g. *Mycobacterium tuberculosis*) grow in matrix-enclosed biofilms in the infected tissues (Lefmann *et al.*, 2006). Early in the process of converting our concepts of acute planktonic diseases into new perceptions of chronic biofilm diseases, the dominant issues were essentially therapeutic. Device-related and other chronic bacterial diseases did not respond to conventional antibiotic therapy, and they rarely resolved as a result of innate or stimulated body defenses; hence, the twin strategies of aggressive debridement and device removal, to

surgically remove all biofilm-infected tissues, evolved in orthopedics (Costerton *et al.*, 2003) and in other medical disciplines (Braxton *et al.*, 2005). More recently, we have realized that the detection of biofilm infections is seriously hampered by the general failure of culture methods to recover and grow biofilm cells from infected tissues, and that this failure of culture methods also affects therapy, in that we lack any rational basis for antibiotic selection.

The general problem in infectious diseases

The culture methods currently in use throughout our medical system were developed by Robert Koch, in Berlin (Koch, 1884), for the detection and characterization of the planktonic bacteria that cause acute epidemic bacterial diseases. When single swimming or floating bacterial cells are transferred to the moist surfaces of agar plates containing suitable nutrients, they replicate to produce colonies, and these colonies can be studied to determine species identity and antibiotic resistance patterns. This very old technology has served us well, and acute epidemic diseases have been largely controlled using culture methods. This is because planktonic bacteria grow well on agar, which provides a ready means for their detection and identification. Moreover, having the causative pathogens in hand facilitates the development of antibiotics and the design of vaccines for their control. Culture methods are still the backbone of the Food and Drug Administration (FDA)-approved diagnostic machinery of our health system and new molecular methods for bacterial detection, using specific antibodies or 16S rRNA gene-specific primers, are only approved for the detection of a small number of pathogens that are difficult to culture (Cloud *et al.*, 2000).

The notion that culture methods have major shortcomings in the diagnosis of biofilm infections emerged gradually, in several medical specialties, but the most definitive work was carried out in connection with otitis media with effusion (OM-E). Even though this chronic infection of the middle ear produced an effusion, containing numerous inflammatory cells and bacteria that could be seen by direct staining, the proportion of positive cultures was so low that putative viral and inflammatory etiologies were seriously considered (Uhari *et al.*, 1995). At this point, Ehrlich and Post mobilized the nascent resources of molecular diagnostics, to show that significant amounts of bacteria DNA were present in the effusions, including the 16S rRNA genes that were characteristic of several species that were occasionally cultured (Post *et al.*, 1995). When it was suggested that the effusions might be full of dead bacteria, Ehrlich and Post showed that the effusions also contained significant amounts of bacterial mRNA (Rayner *et al.*, 1998), which is a very short-lived molecule (< 1 h), whose presence proves that the organisms were not only present at the time of

sampling but also alive and active. These early molecular techniques are essentially research methodologies that are too slow and expensive to be used in routine diagnostics, but the ENT field absorbed this information. Direct confocal microscopic examination of the middle ear mucosa of pediatric patients, and 16S rRNA gene PCR analysis of effusion from the same ear, have now combined to demonstrate that OM-E is a biofilm disease (Hall-Stoodley *et al.*, 2006) that only yields positive cultures infrequently. Similar difficulties with negative cultures, when the clinical signs of infection are obvious, have plagued such fields as urology (prostatitis) and wound management, in which complex multispecies communities yielded only cultures of the few organisms that grew most readily on the media used for culture (Wolcott & Ehrlich, 2008).

The problem in orthopedics

The bacterial infections that affect orthopedic surgery present a favorable exercise in diagnostic accuracy because, with the exception of infections secondary to open trauma, a limited number of species are involved and the detection of organisms in aspirates can often be confirmed by the examination of intraoperative materials obtained during subsequent surgery. Positive cultures are obtained in as few as 30% of cases of septic arthritis in children (Lyon & Evanich, 1999) and attending physicians often treat culture-negative cases empirically, using antibiotics that have been successful in the resolution of culture-positive infections. In cases in which a native joint is inflamed, clinicians often treat with antibiotics and surgical debridement, in the absence of positive cultures, and prosthetic joints are often treated as being infected even though cultures of aspirates and of intraoperative materials are negative. The two-stage revisions of infected joint prostheses recognize the need for the surgical removal of biofilms, and aggressive antibiotic coverage of surrounding tissues and of the replacement prosthesis (Winkler *et al.*, 2008), even in culture-negative cases. Stoodley *et al.* (2008) have also published confocal micrographs showing the consistent presence of biofilms of live coccoid bacterial cells (using Molecular Probes Live/Dead BacLite Kit) in an infected elbow case (Fig. 1) that yielded negative cultures over a period of 5 years, during which the clinical state of the patient necessitated several serious replacement procedures. The confocal data were supported by positive reverse transcriptase-PCR results for bacterial mRNA for *Staphylococcus aureus*.

The orthopedic problem that offers the most dramatic contrast between culture data and modern molecular methods of diagnosis is the tragic problem of the Sulzer acetabular cup. When a critical nitric acid washing step was omitted from the manufacturing process for this device, the microbial biofilms accreted during manufacture were

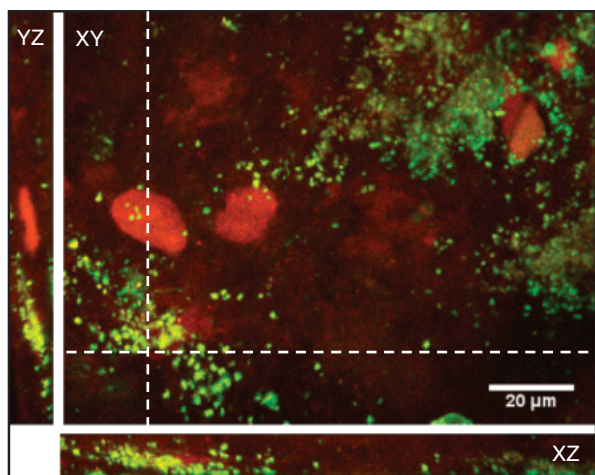


Fig. 1. Confocal micrograph of material stained with the BaCLite Kit. Biofilm clusters composed of aggregates of live cocci (green) are seen on the tissue and in the fluid taken from an elbow that was found to have a biofilm growing on retained tobramycin-impregnated cement following the removal of a failed elbow prosthesis (Stoodley *et al.*, 2008). Aspirates had previously been culture negative and the recurrent symptoms were nonresponsive to antibiotics. The nuclei of host cells were stained red.

retained and, even though ethylene oxide sterilization killed the sessile bacteria, the residual polysaccharides of the matrix increased the colonization potential of these devices. Approximately 1500 cases of 'aseptic loosening' resulted, and this designation was made because the culture results were consistently negative for both aspirates and interoperative specimens (Effenberger *et al.*, 2004). We have examined a subset of eight of these 'aseptic loosening' and, in each case, we have found direct evidence of the presence of bacteria on explants at the time of revision. Figure 2 shows unequivocal evidence of the presence of coccoid bacterial cells on the surface of a culture-negative Sulzer acetabular cup explanted from a case of so-called 'aseptic loosening.' These cells were seen to form slime-enclosed biofilm microcolonies on the plastic surface.

When these acetabular cups were reacted with species-specific FISH probes for *Staphylococcus epidermidis*, the bacterial cells showed fluorescence (Fig. 2, inset), and the cells were seen to be growing in coherent biofilms.

The nature of the problem of culture-negative biofilms

Because the detection of bacteria like *S. aureus* is pivotal in many clinical decisions in orthopedic surgery, and because the presence of methicillin-resistant *S. aureus* (MRSA) can pose intractable problems, it may be valuable to address the culture of the biofilm phenotype of this organism. Extensive studies of the distribution of *S. aureus* in the human female reproduction tract were triggered by the threat of toxic

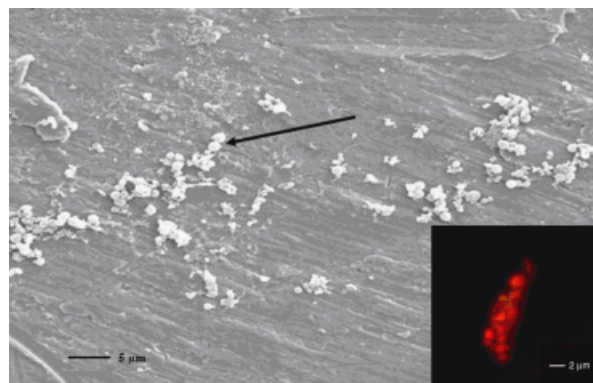


Fig. 2. Coccoid bacterial cells are clearly seen on the surface of a washer from a retention screw used to anchor the Sulzer acetabular cup to the pelvis. The arrow shows a dividing pair of bacterial cells, which indicates that these spheres are living organisms, and the dehydrated remnant of the slime matrix can be seen around the microcolony on the left of this scanning electron micrograph. Scale bar = 5 μm . Inset: shows that the bacterial cells on this surface react with a specific FISH probe for *Staphylococcus epidermidis*. Scale bar = 2 μm .

shock, caused by the secretion of the TSST1 toxin produced by this organism; hence, we explored their detection and characterization using culture methods and new molecular techniques (Veeh *et al.*, 2003). In a survey of 3000 healthy volunteers, using very careful culture techniques in which vaginal swabs were carried to the lab at body temperature and fresh moist plates were used, positive cultures were obtained from 10.8% of these women. This percentage was slightly higher than that found in several previous studies (Wise *et al.*, 1989), probably because of the very careful transfer and processing of the specimens, but longitudinal consideration of the data (Veeh *et al.*, 2003) showed high levels of 'noise' in that individuals yielded positive or negative cultures in an almost random pattern. We examined a subset of 300 subjects, within this large group, using a FISH probe designed to react directly with the 16S rRNA of *S. aureus*, and we found large numbers of cells of this organism in 100% of the subjects. The *S. aureus* cells were mostly present in coherent biofilm microcolonies (Fig. 3), and human epithelial cells bearing individual microcolonies could be identified under phase-contrast microscopy (unpublished data), and placed on the surfaces of agar plates. None of these direct transfers of human cells bearing microcolonies resulted in the formation of colonies on the agar surface.

These data strongly suggested that cells of *S. aureus* that were growing in the biofilm phenotype, when they were transferred to the surfaces of agar plates, fail to produce colonies and are therefore not detected by culture methods. Studies of the proteomes of the biofilm and planktonic phenotypes of *S. aureus* (Brady *et al.*, 2006) indicate that these phenotypes differ profoundly in the genes they express

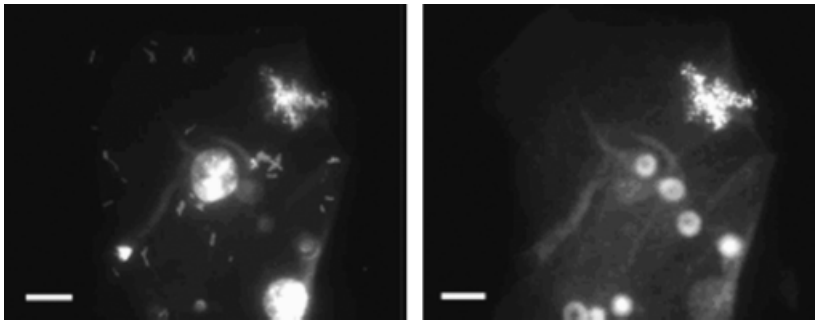


Fig. 3. The human vaginal epithelial cell on the left was reacted with the universal FISH probe eubac 338, and rod-shaped bacilli (probably *Lactobacilli*) fluoresce, as well as the *Staphylococcus aureus* cells in the well-defined microcolony at the upper right. The vaginal cell on the right was reacted with a specific FISH probe for *S. aureus*, and the cells in the well-developed microcolony at the upper right show intense fluorescence. The other fluorescent objects are menstrual red blood cells. The bars indicate 10 μ m.

and, consequently, in the proteins they produce. These phenotypic differences may account for the fact that planktonic cells of *S. aureus* produce colonies on agar, while biofilm microcolonies do not. This notion is supported by the excellent work of Robin Patel's group (Trampuz *et al.*, 2007), who showed that the sonication of orthopedic prostheses before the application of specimens to agar plates released biofilm cells as planktonic cells, and thus increased the number of positive cultures. Similar anomalies have been found in studies (Dowd *et al.*, 2008) that contrast the organisms that are detected using culture techniques with those that are detected using modern molecular methods, in mixed microbial communities in chronic wounds. Molecular methods have replaced culture methods in virtually all branches of microbiology (Hugenholtz *et al.*, 1998), with the notable exception of medical microbiology, and we must realize that biofilms in these natural and pathogenic systems resemble each other so closely that a similar replacement is overdue in orthopedic surgery and in all of Medicine.

Molecular methods for the detection and identification of bacteria

Nucleic acid-based molecular methods for the detection and identification of bacteria begin with the extraction of DNA and/or RNA from the sample to be analyzed. This extraction will be more efficient, and will yield more precise quantification, if the nucleic acids have not been degraded by chemical preservatives or by endonuclease enzymes; hence, fresh or frozen samples yield the best results and rapid processing is essential. Another critical step is getting the nucleic acids out of the bacteria; this can be particularly problematic with Gram-positive bacteria, which have a thick peptidoglycan wall that is difficult to lyse. The problem is compounded when the biofilm is associated with tissue, which itself also needs to be digested to release bacteria that may be attached within surface convolutions or have invaded the tissue itself. We have found that the physical disruption of tissue by bead beating, followed by digestion with lysis buffer (Qiagen AL) and proteinase K (Invitrogen), yielded more consistent results than the use of lysozyme alone, which under-

represented Gram-positive bacteria relative to Gram-negative bacteria (data not shown). Once nucleic acids are extracted and purified, short nucleic acid primers are used to PCR amplify specific DNA sequences. Notably, sequences of the 16S ribosomal DNA that encode the 16S rRNA gene are used because 16S rRNA gene is universal to prokaryotes and is widely used as a phylogenetic 'fingerprint' to identify organisms at the species, genus or phylum level. Other genes of interest such as virulence genes may be probed to identify antibiotic resistance (i.e. *mecA* for MRSA) or sets of genes can be probed for multilocus strain typing, although this is usually done on single isolates. After PCR, the resulting amplicon should contain enough material for analysis. The presence and, in some cases the relative abundance, of amplified gene sequences can be measured using a number of techniques including gel electrophoresis and ionizing spray mass spectroscopy. Quantitative real-time PCR can be used to quantify the starting amounts of DNA by monitoring the amplification during the amplification step. In the case of looking for mRNA to demonstrate not only the presence of a bacterial species but also activity, the mRNA is converted to cDNA by reverse transcriptase before PCR amplification.

It is helpful to visualize a giant forest of mixed bacterial and host DNA that has been extracted from the sample within which small primers seek out corresponding sequences of bases and, when they locate and hybridize with them, produce very large numbers of identical amplicons. The repeated cycling of this process produces very large numbers of identical target sequences termed ampimers or amplicons. The strategies for deciding which genes to amplify, and for selecting methods for the analysis of the amplicons that are produced, have been driven by practical considerations. If one is involved in a leisurely world cruise to study the microbial ecology of the oceans (Ivars-Martinez *et al.*, 2008), speed is not of the essence, and the amplicons can be frozen and analyzed by pyrosequencing over a period of months or years. If one manages a wastewater treatment plant, and is only interested in the detection and identification of a particular invidious bacterium that blocks phosphate removal (Crocetti *et al.*, 2000), a simple and rapid

PCR for that particular organism will suffice. Medical microbiology requires the economical and very rapid detection and identification of a relatively broad range of bacterial and fungal pathogens, and a degree of quantitation that allows the clinician to distinguish between contamination and a genuine infection. These criteria have been elusive, but the recent development of the highly multiplex PCR-based rapid quantitative Ibis technology, which relies on electron spray ionization time of flight mass spectrometry to provide highly accurate nucleotide base ratios (instead of base sequences) of all amplicons, meets these requirements, and will provide the basis for the replacement of culture methods by molecular methods.

Broad-focused molecular methods

In broad-focused methods, the objective is to separate all of the amplicons from the 'forest' of mixed DNA, and from each other, by a physical separation method that is based on variations in their base composition and consequent variations in their molecular weight and/or charge properties. The first such method produced clone libraries from the amplicons, and separated these clones by gradient gel electrophoresis. This denaturing gel gradient electrophoresis (DGGE) method was widely used in microbial ecology, because it was roughly quantitative and produced bands of varying intensities for each set of amplicons, thus providing an approximate estimation of the number of bacterial species present in the sample. This method was used to study the mixed microbial populations present in chronic human wounds (Fig. 4), and we quickly realized that diabetic foot ulcers and venous pressure ulcers contained many more bacterial species than were ever detected by cultures (James *et al.*, 2008).

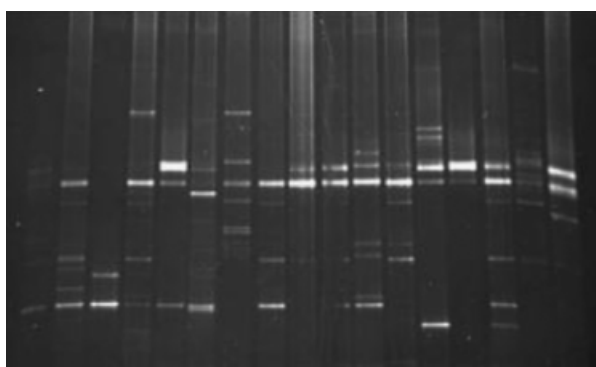


Fig. 4. DGGE of samples from 17 chronic human wounds in which routine cultures never identified greater than three bacterial species, and in which only *Staphylococcus aureus* and *Staphylococcus epidermidis* were usually detected by cultures. DGGE detected as many as 20 separate bands, including those comprised of amplicons of *Propionibacterium* sp. and *Candida albicans*, and clinical management of these chronic wounds was vastly improved using this information.

The distinct bands seen in the gels in DGGE could be analyzed by 454 sequencing, so that the amplicons could be identified at the species level, and then the band could be identified in subsequent samples by its R_f value with reference to migration standards. Variations on these methods were developed, including one in which the amplicons were separated by HPLC, but none of these methods was sufficiently simple and expeditious to provide the rapid diagnosis required for the clinical decisions required in orthopedics. They did, however, establish the fact that cultures were both insensitive and inaccurate, when compared with DNA-based molecular methods.

Narrow-focused molecular methods

All PCR methods use primers with base sequences that match a target region in prokaryotic or eukaryotic DNA, and these primers will always produce amplicons when they 'find' that particular sequence. Thus, in PCR techniques, you find or fail to find what you are looking for. For example, if primers specific for *S. aureus* are used to probe a sample from an infected prosthesis, *S. aureus* will be detected if present, but you will not detect even very large numbers of cells of *S. epidermidis* in the same sample. If you know a medical area very well, and know which bacteria and fungi typically cause infections in this patient population, you can assemble a battery of PCR primers backed up with sequencing data that can provide a much better level of bacterial detections and identification than that provided by cultures (Dowd *et al.*, 2008); however, such an approach relies on the *a priori* selection of targets, and therefore suffers from the 'if you didn't look for it you won't find it' syndrome.

The Ibis molecular method

When the imminent threat of attack with bioterrorism weapons was realized, the Defense Advanced Research Projects Agency of the US Department of Defense initiated an urgent search for new methods for the broad detection and identification of bacteria. Clearly, the existing culture methods were not inclusive of all species and were too slow and cumbersome. Thus, the enemy's selection of a pathogen that was not detected by our well-known cultural paradigms would result in a disastrous failure to diagnose. In response to this call, David Ecker's team, at Ibis, developed a novel strategy in which the amplicons produced by PCR would be weighted by mass spectrometry and their precise weight would be used to calculate their base composition. To provide for the identification of all bacteria, both known and unknown, both pathogen and nonpathogen, multiple sets of primers were designed to detect multiple classes of genes, including those that are highly conserved across entire domains (e.g. 16S and 23S rRNA genes) as well as

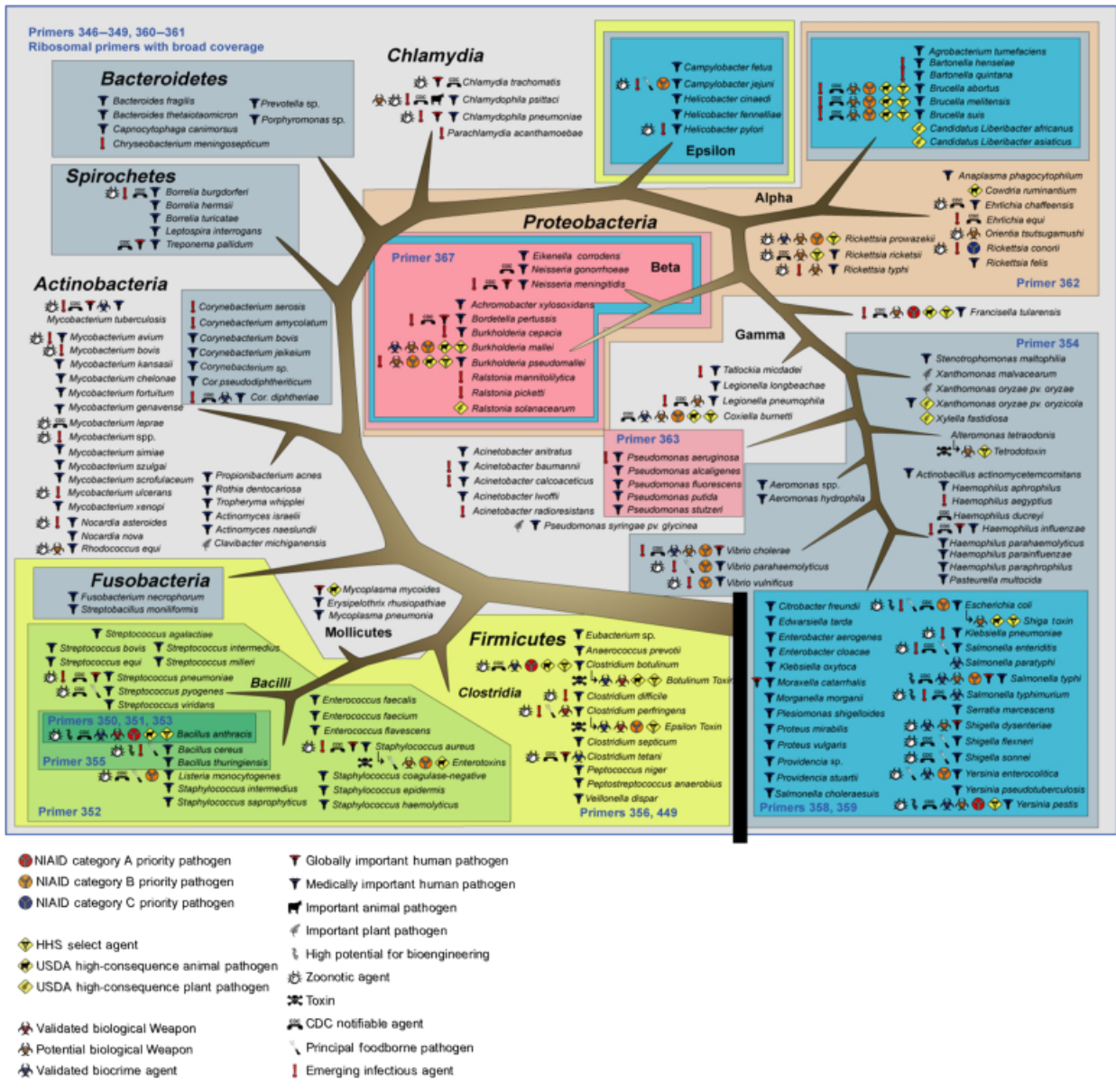


Fig. 5. Diagrammatic representation of the Ibis database in which the base ratios of 286 known species of bacteria are stored and compared, iteratively, with the amplicons produced in the sample by a carefully selected battery of primers. The Ibis universal biosensor system can also detect the presence of fungi and viruses, and can detect the presence of the bacterial genes involved in the resistance to specific antibiotics (e.g. the *MecA* gene present in MRSA).

sequences that are phylum or class specific, and others that are specific to lower taxonomic groupings. Each set of primers is designed to hybridize to a conserved region of a gene that flanks a variable region. Thus, each species that is amplified by each primer pair will produce a different amplicon that is diagnostic or partially diagnostic for that species. By collectively looking at which primers yielded any product, and then characterizing the weight and ultimately the base composition of all the resulting products, it is

possible to precisely determine all those individual species that were present in the specimen. This approach is extremely flexible, allowing the design of different primer sets for a range of applications such as the broad detection of all bacteria, to the much more specific surveillance of influenza strains. No sequencing is required because the base content of the specific variable regions of each amplicon provides the information necessary for making a diagnosis as the system has a look-up database that uses a complex iterative

proprietary algorithm (Ecker *et al.*, 2008) that matches the observed amplicon weights against those of all of the known bacterial pathogens (Fig. 5). If a novel bacterium is present, the system will recognize this because one or more of the amplicon weights will not correspond to any species in the database. In such a case, the system notifies the user that a new species has been identified and what its most closely related relative is.

The precision of this technology does not accommodate any breakage of the amplicons; hence, the sample must be introduced into the specially designed mass spectrograph using a very gentle ionizing method (electron spray ionization), which serves to simply denature the two strands of the amplicon. An internal standard is used to ensure precision in mass determination. The result is that the Ibis universal biosensor detection system can identify the amplicons produced by a carefully designed primer set, with a high degree of accuracy that is stated as a percentage in the 'read out' data and with a sensitivity that detects all organisms present as > 1% of the total microbial population in the sample. The system also detects and identifies fungi and viruses, and detects the presence of the bacterial genes that control resistance to antibiotics. Primer sets can be designed to focus on the pathogens usually seen in a particular medical situation, such as orthopedic infections, so that sensitivity and accuracy can be enhanced in the parts of the bacterial 'tree of life' (Fig. 5) in which the majority of the 'usual suspects' are located. The time required for DNA extraction is short, except in exceptional cases, and the PCR amplification process is rapid and automated, so that the Ibis system can detect and identify all of the bacteria present in a sample in < 6 h, and biofilm cells are detected with the same sensitivity as planktonic cells.

The future

We have initiated prospective double-blinded studies of both suspected infections of total joint prostheses, and of infected nonunions of the tibia/fibula following open trauma, in which we will compare data obtained from cultures with data generated using the Ibis system. Clinical decisions will be based on culture data because the Ibis system is not yet FDA approved, but after the code has been broken, the sensitivity and accuracy of the Ibis system will be compared with that of cultures. In addition, the Ibis data will be considered retrospectively, as a potential basis for clinical decisions, in the light of clinical outcomes and in the light of additional evidence of the presence of bacterial biofilms, such as direct microscopic evidence using FISH probes. If the sensitivity and accuracy of the Ibis system are seen to exceed those of traditional cultures, we will support their adoption for the diagnosis of bacterial infections in all aspects of orthopedic surgery.

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