Culture-Negative Infections in Orthopedic Surgery

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Abstract Laboratory cultures are the main scientific input into the decision-making process that determines the course of treatment for suspected orthopedic infections, just as they constitute the mainstay of the diagnosis of infections in other medical specialties. This situation is archaic because culture techniques were virtually abandoned in Environmental Microbiology (Hugenholtz et al. 1998) many years ago, following the conclusion that <1 % of the bacteria in any natural ecosystem can be recovered by standard cultural methods. Medical Microbiology has clung to

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culture techniques because they detect the bacteria that cause acute infections, with reasonable sensitivity and accuracy, but the time has come to examine both their sensitivity and their accuracy for the detection and identification of bacteria in chronic biofilm infections (Costerton et al. 1999).

1 Culture Methods for Bacterial Detection and Identification

Cultures represent an arcane, but heretofore very useful, technology that remains largely unchanged since they were adopted in Berlin in the mid-1800s (Koch 1884). In this technique, body fluids or tissue specimens are taken from the patient and spread on the surface of an agar medium or grown in a nutrient broth designed to encourage the replication of bacterial cells, until millions of cells of that species either form a macroscopic "colony" on the agar plate or grow planktonically in the broth. The shape and color of the colony help to identify the species of bacteria that have formed the colony, the number of colonies is roughly proportional to the number of cells of that species in the original specimen, and biochemical tests are used to confirm the species identity of the "isolate." The colony can then be "picked" and grown in the presence of antibiotics, at various concentrations, to determine the antibiotic sensitivities of the strain concerned.

This FDA-approved method for the diagnosis of bacterial infections is predicated on the assumption that all of the bacteria of interest to the clinician will grow on the medium that is used, and on the assumption that every bacterial cell will give rise to a separate and distinct colony on the agar surface. In acute bacterial diseases (e.g., "strep throat") these assumptions are reasonable, and even 1/100 dilutions of the specimen will produce hundreds of characteristic colonies that fill the plate and provide both an unequivocal diagnosis and a basis for determining antibiotic sensitivity. The contemporary problem in modern medicine arises from the fact that as many as 80 % of all infections treated by physicians in the developed world are not caused by planktonic bacteria, but are caused by bacteria growing in slime-enclosed biofilms (Costerton et al. 1999). Of special interest to Orthopedic Surgery is the further revelation that virtually all device-related bacterial infections are caused by the biofilm form of bacterial growth (Khoury et al. 1992). The most intuitive of the problems in detecting biofilm bacteria by culture methods derives from the fact that the cells within biofilm communities are bound together by a viscous polysaccharide matrix so that they occur in coherent multicellular aggregates in the specimen (Fig. 1). Obviously hundreds, or even thousands, of bacterial cells bound together in an aggregate will give rise to only a single colony on the agar surface. Robin Patel's group at the Mayo Clinic addressed this problem of bacterial aggregation in orthopedic specimens (Trampuz et al. 2007) and showed that simple sonication breaks up biofilm aggregates, produces some single planktonic cells, and increases the proportion of putatively infected orthopedic prostheses that yield positive cultures.

The aggregation problem is significant, but is dwarfed by the problem posed by the failure of many bacterial cells to grow on the surfaces of the agar media used in

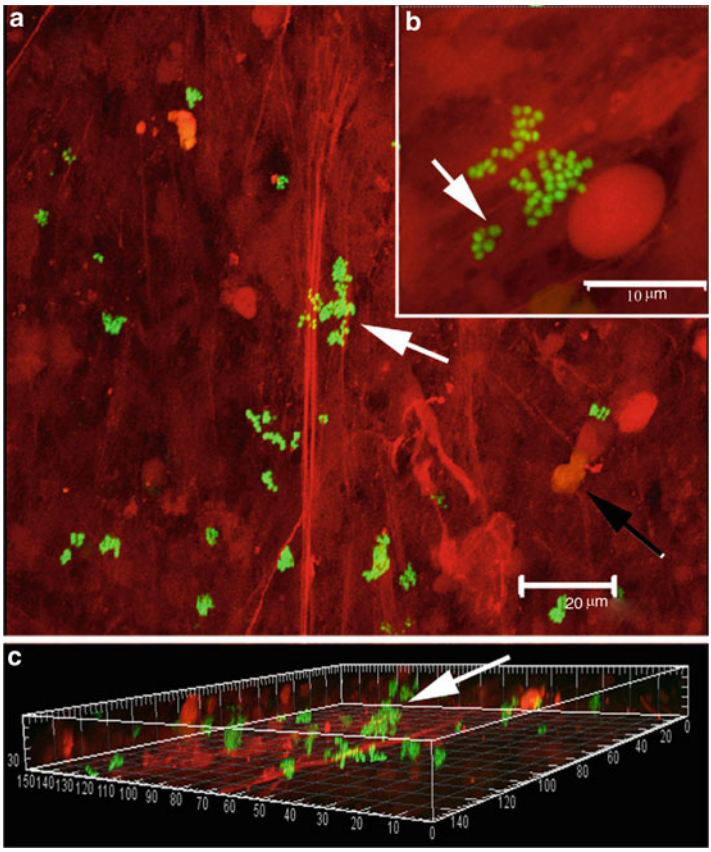

Fig. 1 Material from an infected elbow prosthesis stained with the Molecular Probes live/dead viability kit and examined by confocal scanning laser microscopy (CSLM). Living cells (*green*) of *Staphylococcus aureus* (identified by PCR techniques) are sometimes present as single cells, but they usually form the clusters characteristic of this genus. The clusters (*white arrows*) of bacterial cells are seen in the *x*–*y* projection (A), the high magnification inset (B), and the three-dimensional orthogonal projection (C), and human material (*black arrow*) is seen in the background because it reacts with the propidium iodide in the live/dead kit

routine culture protocols. These media were designed to facilitate the growth of the small number of species that are called "professional pathogens," and that cause the preponderance of acute and epidemic infections in human beings. Most bacterial species fail to grow on the laboratory media used in routine culture protocols, and this phenomenon has led to the virtual abandonment of culture methods in Environmental Microbiology (Hugenholtz et al. 1998). Certainly most anaerobic bacteria cannot produce colonies in routine cultures, and bacteria with fastidious nutrient requirements (e.g., *Propionibacterium acnes*) remain undetected unless special isolation procedures are followed.

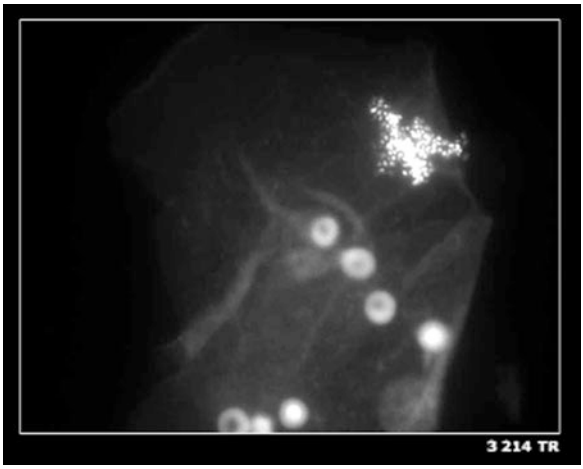

Fig. 2 Fluorescence micrograph of an epithelial cell recovered from the vagina of a healthy human volunteer and reacted with a bacterial 16S FISH probe specifically designed to hybridize only with cells of *Staphylococcus aureus*. This unequivocal evidence of the presence of *S. aureus* biofilms on the vaginal epithelium of 100 % of 300 normal volunteers contrasts sharply with the finding that culture techniques only detected this organism in 10.8 % of the 3,000 volunteers examined in the original study. We concluded that 89.2 % of normal human volunteers, who are heavily colonized by *S. aureus* biofilms, do not yield positive cultures, even when cultures are taken and processed under optimal conditions (Veeh et al. 2003)

But the main cause to failure to grow on routine culture media, and of the consequent failure of culture methods to detect bacterial pathogens, is the fact that bacteria growing in biofilms simply do not produce colonies when they are transferred to the surfaces of agar plates. This phenomenon was first noted when we surveyed 3,000 volunteers for the presence of cells of *Staphylococcus aureus* in their vaginal flora (Veeh et al. 2003), and determined that 10.8 % yielded positive cultures, when swabs were transferred to the laboratory at body temperature and cultured immediately. We then examined vaginal scrapings from a subset of 300 of these volunteers, by modern molecular methods involving the detection of *S. aureus* cells by species-specific fluorescence *in situ* hybridization (FISH) probes (Fig. 2), and found that 100 % were heavily colonized by this organism. FISH probes are unequivocal because they rely on hybridization with prokaryotic (bacterial) 16 S rRNA, which does not occur in human tissues, so we must conclude that all women have this species in their vaginal flora but that only 10.8 % yield positive cultures. Subsequent longitudinal analyses by culture from a subset of the volunteers showed that positive cultures were entirely sporadic and random, leading to the suggestion (Veeh et al. 2003) that cultures were only positive when the coherent *S. aureus* biofilms (Fig. 2) shed planktonic cells that would produce colonies on agar plates. This suggestion was further reinforced when we showed that vaginal epithelial cells bearing large biofilm colonies of *S. aureus* failed to produce colonies when plated on agar media (unpublished data).

2 The Failure of Culture Methods in the Detection of Chronic Biofilm Infections

The simplest and most unequivocal instance of failure to culture, even when bacterial biofilms are present, involves a series of orthopedic device infections. When Sulzer Medical omitted a nitric acid cleaning step in the manufacture of their acetabular cup, several hundred complications known collectively as "aseptic loosenings" occurred. In this condition the prosthesis became loose, and there were multiple symptoms of bacterial infection, but aspirates and intraoperative specimens were uniformly culture negative: hence the name of "aseptic loosening." We (Maale and Costerton) examined eight consecutive explanted culture-negative acetabular cups, and the associated hardware within these hip prostheses, using modern molecular methods for the detection of bacteria. Very large numbers of bacteria were seen when we stained the "ingrowth" tissues at the edges of the acetabular cup with acridine orange (Fig. 3), and these organisms were aggregated in a pattern that proved that they had formed extensive biofilms in these tissues.

Further studies of these culture-negative explanted acetabular cups, using scanning electron microscopy and species-specific FISH probes, showed that the plastic cups were heavily colonized with spherical bacteria cells of *Staphylococcus epidermidis* (Fig. 4) and that the cells reacted with the species-specific 16S FISH probe for *S. epidermidis* (Fig. 4, inset).

The elbow prosthesis shown in Fig. 1 was removed from a patient who endured seven surgical procedures over the course of 5 years, beginning with the placement

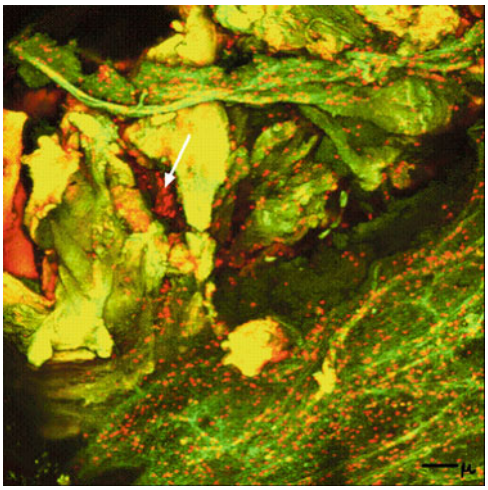

Fig. 3 Confocal light micrograph of an acridine orange stained preparation of "ingrowth" tissue scraped from the surface of a culture-negative Sulzer acetabular cup. Hundreds of orange-stained bacteria are seen to have colonized some elements of the tissue, and a well-developed biofilm aggregate (*arrow*) is seen to fill one of the spaces between tissue components. *Bar* indicates 10 μm

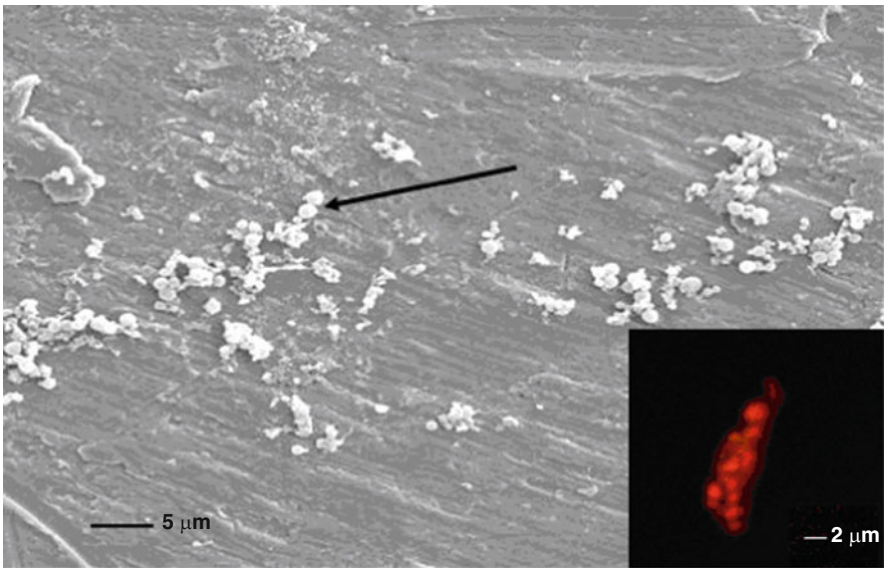

Fig. 4 Scanning electron micrograph of a Sulzer acetabular cup, removed from a case of "aseptic loosening," showing the presence of spherical bacterial cells (*arrow*) in slime-enclosed clusters on the surface of the plastic. The inset shows that these spherical cells react with the species-specific 16S FISH probe for *S. epidermidis*

of a prosthesis and culminating with the removal of this device and the subsequent removal of an associated mass of methyl methacrylate. During this entire period, cultures were consistently negative while the patient's symptoms and the radiolucency of bone in X-rays (Stoodley et al. 2008) clearly indicated that a chronic bacterial infection had caused the failure of this trauma repair. Cultures were positive for *S. aureus* when intraoperative material was sent to the laboratory, from the final surgery, which left the patient with a "flail arm." This case represents a landmark in the diagnosis of chronic bacterial infections in Orthopedic Surgery because aspirates and tissue samples yielded positive results for the presence of *S. aureus* when examined by the reverse transcriptase polymerase chain reaction (RT-PCR), while cultures were consistently negative. We have continued to study individual cases in which cultures of aspirates and of intraoperative materials have been negative, while the attending surgeon suspected the presence of an infection, based on the patient's symptoms and on radiography. Figure 5 shows a bacterial biofilm on the plastic component of a prosthetic ankle, from a patient whose aspirates and intraoperative specimens were culture-negative, but whose prosthesis was clearly infected by biofilm-forming bacteria. This image shows the presence of living bacterial cells, while parallel analysis by PCR-ESI-TOF-MS showed the presence of *S. aureus*, and FISH probe analysis of the same sample showed the presence of large numbers of cells of this organism.

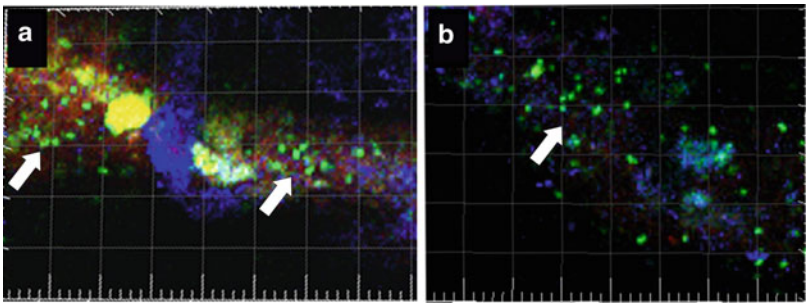

Fig. 5 Confocal micrograph of a bacterial biofilm that had formed on the plastic component of a prosthetic ankle joint, which never yielded positive cultures, from aspirates or from intraoperative materials. The preparation has been reacted with the Molecular Probes live/dead kit so that living bacteria are *yellow*/*green* and dead bacteria are *red*, and these bacterial cells are seen to comprise an extensive matrix-enclosed microbial community in which most of the bacteria are alive. The *bar* indicates 10 μm

3 The Solution to the Problem of Negative Cultures

When microbial ecologists were faced with the problem of the lack of sensitivity and accuracy of classic culture techniques, in bacterial population analyses in natural ecosystems, they turned to DNA-based molecular techniques (Hugenholtz et al. 1998). Two streams of techniques soon developed, in that broad low-resolution techniques (e.g., DGGE and D-HPLC), were used to determine how many species were present in mixed populations, while focused high-resolution methods (e.g., DNA sequencing) were used to identify individual species. The molecular bases of these broad and focused techniques are discussed, in detail, in our recent review in FEMS Immunology and Medical Microbiology (Costerton et al. 2010). The DGGE technique was used to determine how many bacterial species were present in chronic wounds (James et al. 2008), identifying as many as 22 bacterial species in wounds (diabetic foot ulcers) that yielded positive cultures only for one or two commonly isolated pathogens (e.g., *S. aureus*). None of these molecular methodologies could have met the requirements of clinical diagnostic facilities, between 1980 and 2007, because none could provide rapid data and because the vital component of antibiotic resistance patterns was still lacking.

While they could not yet provide a routine technical platform for rapid and accurate diagnostics, DNA-based molecular techniques were used to establish the bacterial etiology of otitis media with effusion (OME), and several other chronic biofilm infections. Ehrlich and Post proved that large amounts of bacterial DNA were present in OME, although most cultures were negative (Post et al. 1995), and they even showed that the infected tissues contained short-lived bacterial messenger RNA (mRNA), to establish that these bacteria were alive and metabolically active (Rayner et al. 1998). Ultimately, these infections were established as classic biofilm infections when they displayed matrix-enclosed bacterial colonies upon imaging, in

both animal models of infections (Ehrlich et al. 2002) and human middle-ear infections (Hall-Stoodley et al. 2006). Cultures in both of these studies were usually negative Dowd et al. (2008) have used a combination of PCR methods with pyrosequencing to show that many bacteria and fungi are present simultaneously in chronic wounds, but that cultures only detect a small fraction of these pathogens. This information has produced dramatic improvements in treatment (Wolcott and Ehrlich 2008) because antibiotic therapy can now be directed at the control of all of the pathogens (e.g., *Candida albicans*), and all can be suppressed or killed. These studies suggest that the suppression of one pathogen amongst many may lead to the resurgence of the organisms that have not been detected or treated, and to the prolongation of infections that are already chronic and refractory.

All of these studies of device-related and other chronic bacterial infections have produced a burgeoning mass of evidence that culture methods are both inaccurate and insensitive in the diagnosis of bacterial infections. This realization has crystallized, in fields as dissimilar as ENT and Orthopedics, but cultures have persisted as the gold standard because they can (ideally) provide an answer in 24 h and an antibiogram in 48 h. Some PCR methods (Cloud et al. 2000), and some methods based on antibodies (Brady et al. 2006), provide very rapid diagnosis, but we only "find what we are looking for" and we do not get a global picture of all of the organisms that are present. So clinical medicine is poised and waiting for a method for the accurate and sensitive detection and identification of bacterial pathogens, and an equally accurate means of determining their sensitivity to antibiotics.

4 The IBIS PLEX-ID

The bioterrorism defense community has an urgent need for the rapid and accurate detection and identification of bacterial pathogens, and they have fostered the development of the Ibis universal biosensor. This technology is based on the "weighing," by mass spectroscopy (Ecker et al. 2008), of PCR-amplified bacterial DNA in samples, and the use of a complex algorithm to match the base ratios in these amplicons with those of many hundreds of bacterial species whose base ratios are stored in an interactive database (Fig. 6).

We have begun to compare the diagnostic capabilities of the IBIS technology with those of routine cultures, and, while we cannot yet predicate our treatment based on the Ibis system because of FDA restrictions, the advances in sensitivity and accuracy are patently obvious. Table 1 shows the data from four cases that were analyzed before we started our very extensive blinded prospective clinical trials of putative infections of total joints and of infected nonunions.

In each case in which culture methods had produced a diagnosis (MRSA or MRSE), the Ibis technique confirmed that diagnosis by detecting *S. aureus* or *S. epidermidis*, and the *Mec A* methicillin resistance gene cassette. In all of these positive culture cases, the Ibis detected additional organisms, and, in cases 010609 and 122308, these data would have changed the strategy for antibiotic therapy. In the

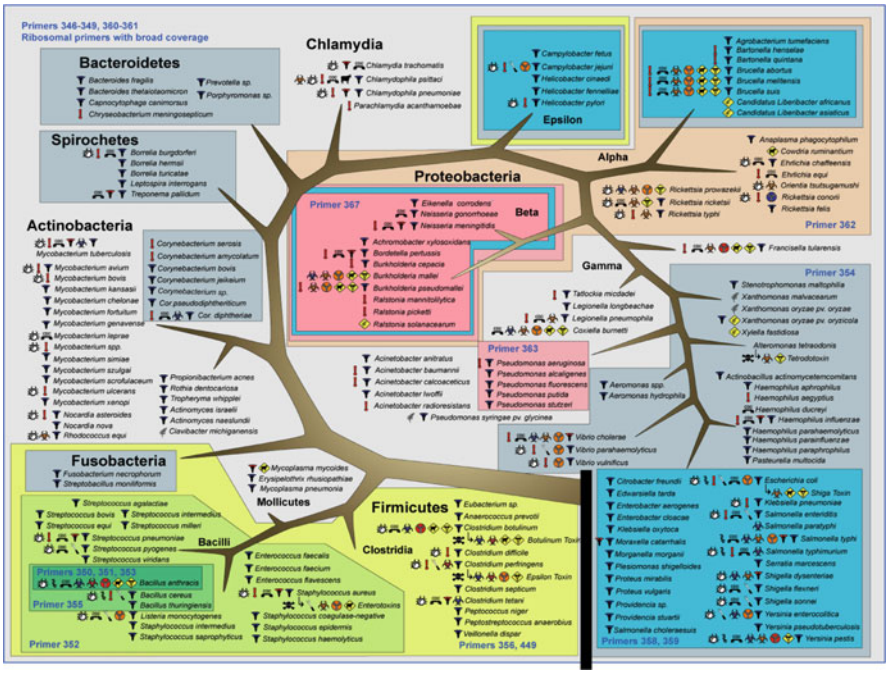

Fig. 6 The IBIS database contains the base ratios of thousands of known bacterial pathogens, and the base ratios of the bacteria in samples can be determined and matched to those in the database, to determine the presence of any of these organisms. If an organism is not present in the database, it will be detected but not identified, and the relative prevalence of all organisms is established by the number of genomes present in the sample. The Ibis system also detects the genes responsible for antibiotic resistance, so a molecular antibiogram is provided in the 6-h time frame necessary for this analysis

Table 1 Orthopedic cases in which the IBIS technique was compared with routine cultures

Ortho case	Culture/Gram stain	Amplicon number and confidence of match	Prevalence = genomes/well	Identification of bacteria + antibiotic resistance
120308	MRSA	1 = 100 %	3,889	*S. aureus*
		2 = 92.4 %	452	*S. epidermidis*
		3 = 100 %	8,184	Methicillin Res.
121908	Culture neg.	1 = 90.8 %	10,739	*S. warneri*
	Few Gram + cocci	2 = 100 %	11,429	*S. capitis*
		3 = 88.2 %	1,460	*P. acnes*
		4 = 100 %	1,777	Methicillin Res.
010609	MRSA	1 = 87.9 %	267	*S. aureus*
		2 = 96.7 %	124	*S. epidermidis*
		3 = 94.8 %	641	*E. faecalis*
		4 = 99.7 %	7,474	*B. cereus*
		5 = 100 %	714	Methicillin Res
122308	MRSE	1 = 99.3 %	6,315	*S. epidermidis*
		2 = 97.6 %	2,058	*S. capitis*
		3 = 99.6 %	900	*B. cereus*
		4 = 100 %	20,236	Methicillin Res.

culture-negative case, very large numbers of methicillin-resistant coagulase-negative Staphylococci were found by the Ibis technology, and a chronic biofilm infection was clearly present. In this case a Gram-positive pathogen (*P. acne*) was also present and would have triggered appropriate antibiotic therapy if the IBIS system was approved for bacterial diagnosis.

5 Summary

Culture methods are no longer used for the detection and identification of bacteria, in many fields of Microbiology (e.g., Microbial Ecology) that can accommodate the leisurely pace of pyro-sequencing and other DNA-based molecular methods. Where these methods have been used to detect and identify bacteria in human infections, they have proven to be more accurate and more sensitive than culture methods, but their slow pace and high cost have prevented their adoption for routine diagnosis. Some very rapid diagnostic methods, based on PCR amplification or on reaction with specific antibodies, have gained some acceptance, but these highly focused methods only look for specific organisms and cannot detect all of the bacteria present in a sample. We have examined a new mass-spec-based technology for the detection and identification of bacteria that is based on the base ratios of segments of several critical bacterial genes, and that is very rapid (<6 h) because it does not involve sequencing of these bases. This Ibis technology detects and identifies bacteria with much more sensitivity than cultures, and it solves the dual problems posed by biofilms, in that cells in clusters are detected individually, and in that cells that fail to grow on culture media are detected quantitatively. The Ibis technology also detects the major bacterial genes that control antibiotic resistance, so bacteria can be detected and identified in 6 h, and their antibiotic resistance profiles are also known in this same short timeframe. We suggest that a systematic comparison of the Ibis technology with culture methods should be undertaken, with full training of clinicians in the interpretation of molecular data, with the intent of replacing cultures with molecular techniques in the immediate future.

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