



Proteomics: A Revolution in the Evaluation of Gastrointestinal Microflora

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Recent research regarding the gastrointestinal microbiome has irrefutably confirmed the fact that the microbial inhabitants of the gastrointestinal tract, and their astonishing scope of metabolic activities, are at the very core of health and numerous disease processes. It is also clear that clinical microbiology testing should be optimized to address the relative abundance of all bacterial species present in a stool specimen. To that end, culture remains the current standard of practice in high-complexity clinical microbiology testing. A revolutionary methodology has been developed and is employed at Doctor's Data to markedly expand our ability to identify species and even subspecies of gastrointestinal bacteria and yeast. This article will

summarize the pros and cons of current methodologies used to study gastrointestinal flora, with emphasis on the application of a proteomic-based method that optimizes the superior specificity of highly sensitive culture-based comprehensive stool analysis.

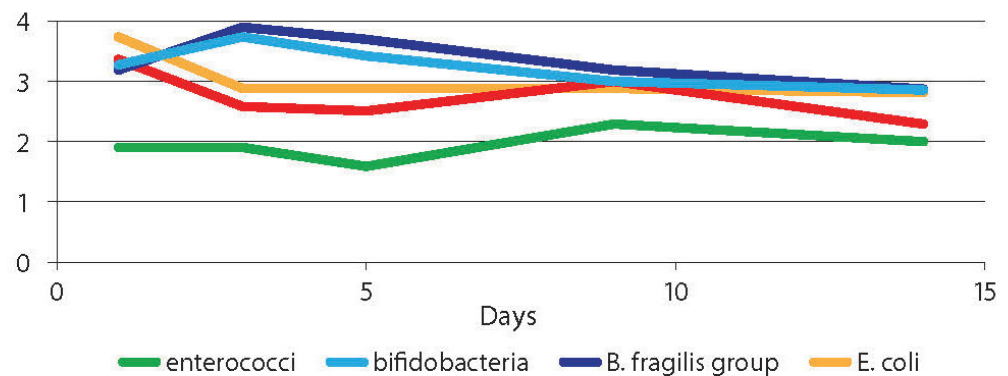


Figure 1. Growth of beneficial/expected bacteria from Cary-Blair transport media over time.

Culture-Based Testing

Culture growth-based testing remains the international standard for clinical microbiology testing for stool specimens. Culture enables identification of living, metabolically active microorganisms, as dead bacteria do not contribute to the metabolomic environment of the gut. Further, live, pure bacterial isolates are required for antibiotic (AB) susceptibility testing. Recent research from Boston University indicates that gastrointestinal virus-derived phages harbor numerous antibiotic AB resistance genes that can be transferred to bacteria. However, simply detecting one of the hundreds of AB resistance genes in a bulk stool specimen does not directly indicate that an AB resistance gene is actually present or expressed in a particular microbial species. This requires culture to obtain a pure isolate of the pathogenic species of concern. What's more, AB resistance genes may be "silent," or not expressed or activated by specific promoters, and more complicated and genetically diverse mechanisms of antimicrobial resistance remain elusive. The bottom line is that genotyping for functioning AB resistance genes has not been validated for stool specimens for clinical purposes.

Susceptibility to antibiotics must be directly performed on pure, cultured isolates using standardized microbiological techniques according to the Clinical Laboratory Standards Institute's guidelines, and validated in the specific laboratory. Clinicians should not hesitate to ask the laboratory if such criteria are adhered to.

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Misconceptions about Culture

Several exaggerations have been perpetuated regarding the viability and stability of bacterial populations during transport to the laboratory after specimen collection. The recommended transport media for stool specimens, Cary-Blair media, is buffered to prevent shifts in pH and has a very low nutrient content—not “loaded with sugar”—that suspends growth of stool bacteria and inhibits the growth of other bacteria. In addition, the transport media has a low agar content to enhance viability of pathogenic species.

Critics of culture have also suggested that anaerobic bacteria cannot survive sampling and transport to the laboratory. While it is true that some strict anaerobes are not yet able to be cultured, it should be noted that Cary-Blair media contains a reducing agent that markedly lowers the oxygen content and actually favors anaerobes. As shown in Figure 1, stool specimens were collected and held in the transport media for up to 14 days prior to culture under anaerobic conditions at Doctor's Data, Inc. (DDI). It is clearly evident that all of the levels of beneficial and expected strict anaerobes and facultative anaerobes that DDI reports are stable, with respect to clinical significance, out to 10 days after collection. This is particularly impressive for the strict anaerobes (*Bifidobacterium spp.* and the *Bacteroides fragilis* group). Most specimens are received within 48 hours of specimen collection.

Understanding *Clostridium* Results

DDI reports *Clostridium spp.* under Expected/Beneficial bacteria because a normal gastrointestinal tract can contain a relatively high amount of the approximate 100 different species of *Clostridium*, while only 4 are overtly pathogenic. Although *Clostridium* are labile in an oxygenated environment, we are able to grow the strict anaerobes under our standard anaerobic culture conditions because all *Clostridia* produce virtually indestructible spores that become vegetative microorganisms when appropriately cultured. Even under standard anaerobic conditions, we occasionally see growth of *Clostridium difficile*. When this occurs, the isolates are reflexively tested at no charge for the PaLoc gene locus using an FDA-cleared gene amplification platform (LAMP). The PaLoc locus is required for production of toxins A and B, and all toxigenic *C. difficile* contain the PaLoc gene segment. *C. difficile*-associated disease occurs almost exclusively when beneficial bacteria are suppressed after exposure to broad-spectrum antibiotics, and only occurs when the bacteria contain the PaLoc gene locus. Therefore, one need not treat *C. difficile* that do not contain the PaLoc gene. For a complete and discriminating breakdown of *Clostridia* species, DDI offers a Comprehensive Clostridium Culture test that is optimized specifically for growth of *Clostridium* species.

High-complexity clinical microbiology, as performed at DDI, entails much more than one might envision. Upon receipt, the stool specimens in the transport media are mixed and 1 gram of stool is streaked onto a quadrant of 10 agar plates (5 aerobic, 3 anaerobic, 1 microaerophilic, and 1 yeast plate that contains chromogenic media). After plate-specific variable incubation times, growth for each colony that grows is scored and individual colony forming units (CFUs) are subcultured in selective agars and media, guided by the results of gram staining and basic biochemical discrimination. After the second incubation, the pure isolated bacteria and yeast are ready for identification.

Identification of Bacteria and Yeast from Pure Isolates

Identification by Phenotype

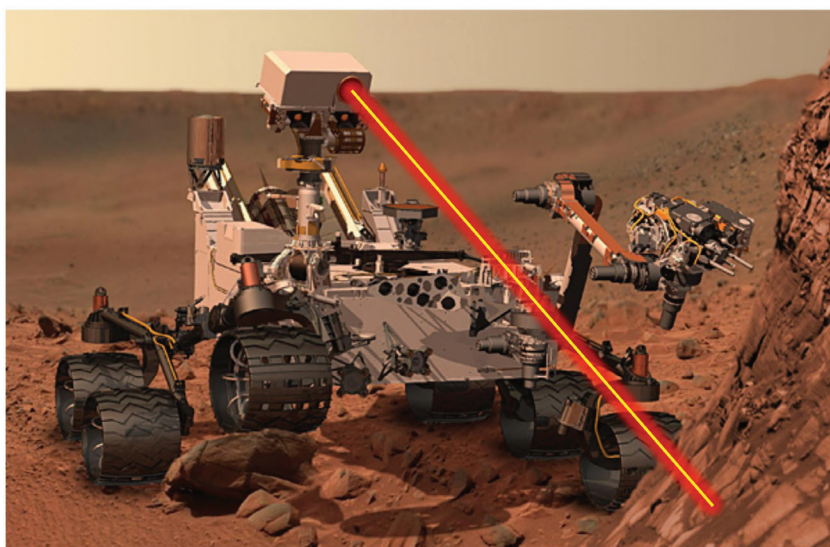
In the past, microorganisms were identified manually by phenotypic identification, a vast array of time-consuming biochemical reactions. Eventually, technological advances provided automated systems such as Vitek-2™ to perform phenotypic identification. Such automated identification instruments were considered to be state-of-the-art and markedly reduced manual labor, human error and judgment, and time to identification, and are currently used in most institutional and commercial clinical microbiology laboratories. They are limited, however, with respect to the number of microorganisms that can be confidently identified. If a microorganism is not identifiable with greater than 90% confidence, additional time-consuming off-line testing is required to obtain a computer-assisted, best-fit identification. These

methodologies for identification of microorganisms can take as long as 6 to 24 hours and prolong the time before beginning subsequent antimicrobial susceptibility testing. Despite these inherent limitations, automated instruments are still invaluable for susceptibility testing with commonly prescribed pharmaceutical antimicrobials.

Identification by Proteomic Analysis

Proteomics entails the identification and characterization of proteins in cells, tissues, or organisms. This allows identification based on each microorganism's unique ribosomal protein fingerprint.

Doctor's Data utilizes a revolutionary state-of-the-science proteomic methodology for rapid, accurate, and reliable identification of thousands of gastrointestinal bacterial and yeast species, and even subspecies. The methodology, matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF MS), has been recognized by a share of the 2002 Nobel Prize in Chemistry. MALDI-TOF MS has been used to study a host of macromolecules from a wide variety of biological matrices, just as similar methods of detection have been used by NASA on the Mars Curiosity Rover in an effort to determine whether the planet had been hospitable for microbial inhabitance.



Mars Curiosity Rover

generate the mass spectrum; and (5) comparison of the unknown spectrum to an extensive database.

In the laboratory, a small portion of a pure isolate from selective subculture is embedded within a saturated liquid matrix on a 96-spot target plate. The matrix solution crystallizes and charges the proteins (primarily +1), then the dried mixture of matrix and proteins is vaporized by nanosecond-duration laser pulses (MALDI) that release the ionized protein molecules that are accelerated in an electric field within a vacuum. Proteins fly through the mass analyzer (TOF) at a rate that is inversely proportional to the mass of the protein. The proteins reach the mass spectrometer over a span of to about 0.01 to 1 millisecond—the timescale to analyze the ions produced by a single laser pulse permits acquisition of data from multiple laser pulses for a specimen. The mass spectrum of the unknown pure isolate is compared to a high-quality reference database of spectra generated from quality-controlled collection strains. The database contains about 7,000 microorganisms and is growing at about 400 more per month, although not all of the microorganisms are derived from the gastrointestinal tract. A goodness of fit is ranked and a log score

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MALDI-TOF MS has recently been applied to and established as an invaluable technique in clinical microbiology. In brief, MALDI-TOF MS enables rapid and accurate identification of normal and pathogenic gastrointestinal microorganisms based upon their signature high-abundance proteins.

There are 5 steps in the MALDI-TOF MS identification process: (1) ionization of proteins from a pure isolate; (2) separation of vaporized, ionized proteins that have different masses; (3) detection of the number of the different ionized proteins; (4) collection of data to

threshold is applied for correct identification at the species level (2.300-3.000), secure genus/probable species (2.000-2.299), or possible genus (1.700-1.999). Inclusion in the database requires ≥ 10 independent reference spectra with a reference score ≥ 1.9 for a given bacteria or yeast. To date, using MALDI-TOF MS, DDI is able to identify more than 400 bacterial species and 125 yeast species that were not identifiable using the previously employed automated phenotypic instruments used by most clinical microbiology labs. In addition, we have validated secure identification of yeast at the species level at a ranking score of just 1.800, and have been able to identify some bacteria at the subspecies level. Amazingly the MALDI-TOF MS identification process takes less than 1 minute per isolate.

Based on work in her research lab and the plethora of published studies regarding MALDI-TOF MS, Dr. Sandra Richter, Director of Bacteriology in the departments of Clinical Pathology and Molecular Pathology at The Cleveland Clinic, stated that "It is clear that this is a giant step forward in the accuracy and speed of organism identification routinely reported by the lab." (*CAP Today*, 2013;27[1]).

Consistent, Validated Results

Accuracy and speed are important, but if a clinician sends a specimen to several labs will they get the same results? That is a significant issue when laboratories are not using standardized, validated test platforms. The results of an international, multicenter trial to evaluate the reproducibility of a commercially available MALDI-TOF MS platform, the Bruker Biotyper™, were presented at the 19th European Congress of Clinical Microbiology and Infectious Diseases in Helsinki in 2009. Sixty blind-coded bacterial specimens were sent to 8 international laboratories, for a total of 480 specimens. A concordance rate of 98.8% was achieved among the labs. Not only were the samples correctly identified across laboratories, but the reliability of identification was very high, as 97.3% of all log score values were >2 . What's more, the MALDI-TOF MS approach has an impressive capability to differentiate closely related species from a genus such as *Bacillus*. The genus *Bacillus* is one of the largest, most ubiquitous genera of bacteria, with about 65 valid species.

OVERALL CONSIDERING ACCURACY, INTERLABORATORY REPRODUCIBILITY, COST AND THE RAPIDITY OF IDENTIFICATION, THE MALDI-TOF MS PROTEOMIC APPROACH IS BEST SUITED FOR ROUTINE COMPREHENSIVE STOOL ANALYSIS.

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