SPECIAL EDITION

THE STANDARD

DOCTOR'S DATA INC.
SCIENCE + INSIGHT

News and scientific learning from Doctor's Data, Inc., a clinical laboratory providing accurate, innovative specialty testing for over 40 years.

Proteomics: A Revolution in the Evaluation of Gastrointestinal Microflora

BY DAVID QUIG, PHD

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, and a revolutionary methodology has been developed and is employed at Doctor's Data to markedly expand our ability to identify species and even sub-species 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.

Culture remains the current standard of practice in high complexity clinical microbiology testing.

Culture

Culture growth-based testing remains the international standard for clinical microbiology testing for stool specimens. Culture enables identification of living metabolically active microorganisms. 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- such would require culture to obtain a pure isolate of the pathogenic species of concern. Further AB resistance genes may be "silent" (not expressed /not activated by specific promoters), and more complicated and genetically diverse mechanisms of antimicrobial resistance have remained elusive. The bottom line is that genotyping for functioning AB resistance genes has not been validated for stool specimens for clinical purposes and susceptibility to antibiotics

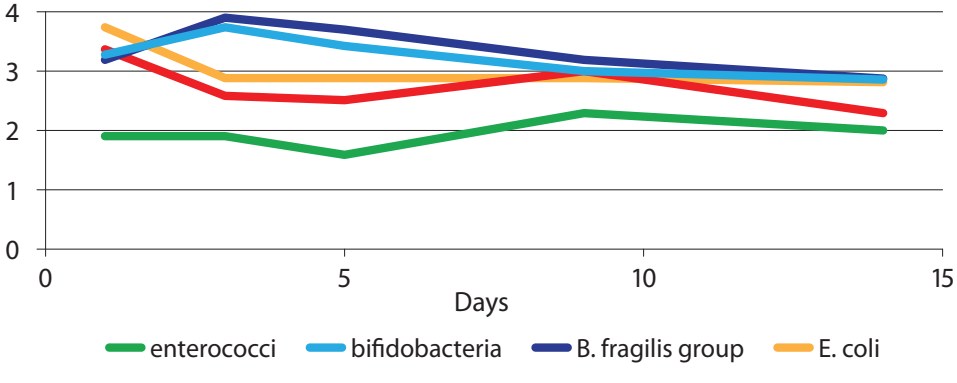


Figure 1. *Growth of beneficial/expected bacteria from Cary-Blair transport media over time.*

CONTINUED ON NEXT PAGE

In This Issue

KNOWLEDGE SPOT

Why did DDI choose MALDI-TOF mass spectroscopy instead of a PCR-based technology?

Read all about it on the back page.

Proteomics: A Revolution in the Evaluation of Gastrointestinal Microflora

CONTINUED FROM COVER

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.

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 exaggerated that anaerobic bacteria cannot survive sampling and transport to the laboratory. While it is true that some strict anaerobes are not able to be cultured to date, it should be noted that the Cary-Blair media contains a reducing agent that markedly lowers the oxygen content and actually *favors* anaerobes. Proof of concept is provided 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/expected strict anaerobes and facultative anaerobes that DDI reports are stable, with respect to clinical significance, out to ten days after collection. Such is particularly impressive for the strict anaerobes (*Bifidobacterium spp.* and the *Bacteroides fragilis* group). Most specimens are received within 48 hours of specimen collection. 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* (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 occasional see growth of *Clostridium difficile*- when such occurs the isolates are reflexively tested (at no charge) for the gene locus (PaLoc) using an F.D.A. cleared gene amplification platform (L.A.M.P.). The PaLoc locus is required for production of toxins A and B. All toxigenic *C. difficile* contain the PaLoc gene segment. *C. difficile* associated disease occurs almost exclusively after beneficial bacteria are suppressed after exposure to broad spectrum antibiotics, and only occurs when the bacteria contain the PaLoc gene locus. 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 one gram of stool is streaked onto a quadrant of ten different agar plates (5 aerobic, 3 anaerobic, 1 microaerophillic, and 1 yeast plate that contains chromogenic media). After plate-specific variable incubation times growth for each and every colony that grows is scored (0-4+), and individual colony forming units (CFU) are sub-cultured (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 a vast array of time consuming biochemical reactions (phenotypic identification). Eventually technological advances provided automated systems (e.g. Vitek-2™) to perform the phenotypic identification processes. Such automated identification instruments were considered to be the state-of-the-art, markedly reduced manual labor, human error/judgment and time to identification, and are currently used in most institutional and commercial clinical microbiology laboratories. However they are limited 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

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 sub-species.

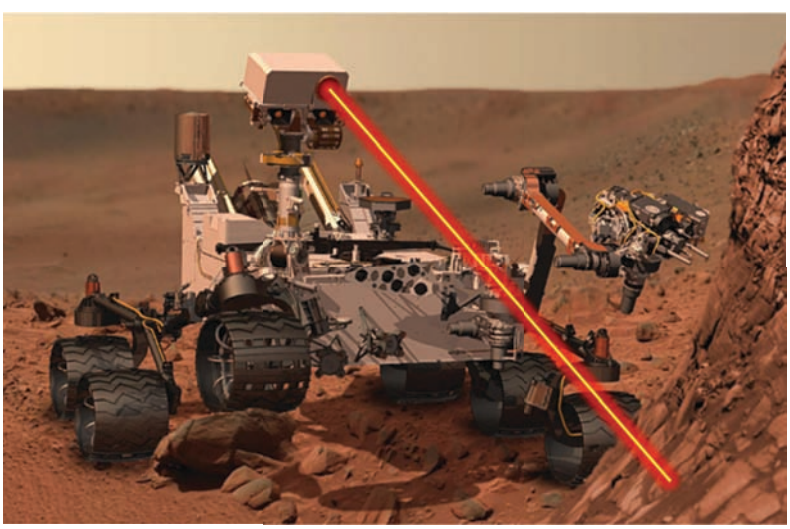
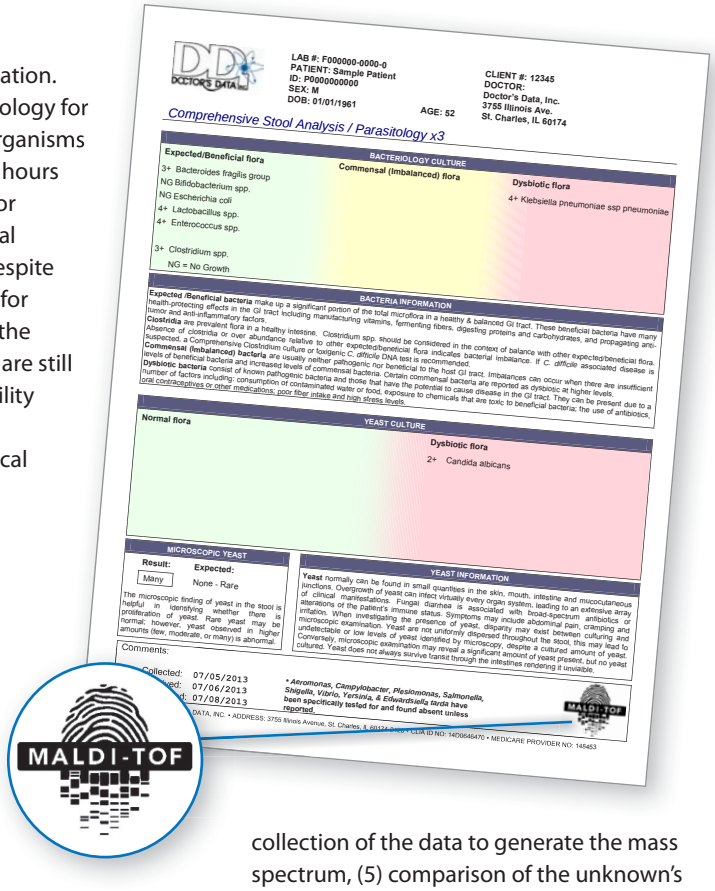


Figure 1. *Mars Curiosity Rover*

assisted best fit identification. The automated methodology for identification of microorganisms can take as long as 6-24 hours and prolongs the time for subsequent antimicrobial susceptibility testing. Despite the inherent limitations for microbial identification the 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. Microorganisms have unique ribosomal protein "fingerprints." 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 sub-species. The methodology, Matrix- Assisted Laser Desorption/Ionization Time of Flight Mass Spectroscopy (MALDI-TOF MS) has been recognized by a share of the 2002 Noble Prize in Chemistry. MALDI-TOF MS has been used to study a host of macromolecules from a wide variety of biological matrices, and similar methods of detection have been used by NASA on the Mars Curiosity Rover (Figure 1) in effort to determine if the planet had been hospitable for microbial inhabitance. MALDI-TOF MS has recently been applied to, and established as an invaluable technique in clinical microbiology. In a nutshell, MALDI-TOF MS enables rapid and accurate identification of normal and pathogenic gastrointestinal microorganisms based upon their signature high-abundance proteins.

There are five steps in the MALDI-TOF MS identification process (1) ionization of proteins from a pure isolate, (2) separation of the vaporized, ionized proteins that have different masses, (3) detection of the number of the different ionized proteins, and collection of the data to generate the mass spectrum, (5) comparison of the unknown's spectrum to a huge data base.

In the laboratory a small portion of a pure isolate from selective sub-culture is embedded within a saturated liquid matrix on a 96 spot target plate- the matrix solution crystallizes and charges the proteins (primarily +1). 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 detector (mass spectrometer) over a time span of to about 0.01-1 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 (about 7,000 and growing at about 400/month)- not all of the microorganisms in the data base are derived from the gastrointestinal tract. A "goodness of fit" is ranked and a log score 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 vast and growing 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 has been able to identify over 400 bacterial species and eight yeast species that were not identifiable using the previously employed automated phenotypic instruments that are 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 one minute per isolate.

Based on work in her research lab and the plethora of published studies regarding MALDI-TOF MS, Dr. Ritcher (director of bacteriology in the departments of Clinical Pathology and Molecular Pathology, Cleveland Clinic) stated that "It seems like a no-brainer to bring this technology into the clinical laboratory," and "It is clear that this is a giant step forward in the accuracy and speed of organism identification routinely reported by the lab." (*College of American Pathologists Today*, January, 2013, vol. 27 no.1).

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 (Bruker Biotyper™) were presented at the 19th European Congress of Clinical Microbiology and Infectious Diseases in Helsinki, Finland in May of 2009. Sixty blind-coded bacterial specimens were sent to eight different international laboratories. A concordance rate of 98.8% was achieved among labs for the 480 blind-coded specimens. Not only were the samples correctly identified across laboratories, but the reliability of identification was also very high as 97.3% of all log score values were > 2.

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 (about 65

CONTINUED ON PAGE 4

Proteomics: A Revolution in the Evaluation of Gastrointestinal Microflora

CONTINUED FROM PAGE 3

valid species) and, the nonpathogenic strains have the highest potential to produce false positive results when analyzed by 16S rRNA sequence analysis due to the high degree of conservation among *Bacillus* species. Sixteen isolates of putative *B. pumilus* spores were isolated from various NASA spacecraft assembly facilities (10-100K clean rooms) and attempts to identify the species included a standard biochemical platform, MALDI-TOF MS and 16S rDNA sequencing. The MALDI-TOF MS protein profiling platform (Bruker Biotyper™) was more accurate than the biochemical platform, more discriminating than the 16S rDNA sequence analysis platform, and complimented the results of genetic methodologies that have greater discriminatory power than 16S rDNA (gyrB sequence analysis and DNA-DNA hybridization). Considering accuracy, interlaboratory reproducibility, cost and the rapidity of identification the proteonomic approach (MALDI-TOF MS) is best suited for routine comprehensive stool analysis.

Identification by PCR

Polymerase chain reaction (PCR) is a relatively old biochemical technology that is used in molecular biology to amplify (exponentially) select segments of DNA and has been recognized by a share of the 1993 Noble Prize in Chemistry. After amplification several different means of analysis have been applied in order to identify the preselected target segment of DNA including Real-Time Detection PCR (RTD-PCR), PCR-ELISA, and PCR. RTD-PCR platforms are able to detect sequence-specific PCR products as they accumulate in "real-time" during the PCR amplification process, and have proven utility for clinical microbiology use in hospital settings for rapid (<3 hours) identification of causative enteric bacteria associated with serious diarrheal diseases. The expeditious nature of the RTD-PCR platforms is due to the fact the bacteria do not have to be cultured. Recent studies have indeed demonstrated that a three-hour RTD-PCR platform exhibited 92% (96/104) sensitivity compared to standard culture and phenotypic identification of *Campylobacter, Salmonella, Yersinia* and *Shigella* species. The investigators acknowledged that, paired with reflexive culture testing, the RTD-PCR platform utilized provides improved care for patients with acute infectious diarrheal disease when time is of the essence. RTD-PCR has similarly been applied for the rapid detection of other enteric pathogens such as *Clostridium difficile*, *Escherichia coli* (EHEC) and *Vibrio* species. Rinttilä and colleagues designed an extensive set of Real-time PCR assays targeting a large group of predominant and pathogenic human gut microbial species. They demonstrated that their real-time PCR platform is very sensitive and precise for quantitative detection of human pathogens from fecal samples. Importantly, using fecal samples spiked with various amounts of target bacteria they demonstrated that detection limits could be obtained that were between 6×10^3 (6,000) (*H. pylori*) and 6×10^4 (60,000) (*Clostridium difficile* and *Campylobacter jejuni*) CFU/g (Rinttilä TA et al. 2004. J Appl Microbiol 97:166-77).

Method	Detection Limit (CFU/gm)	Linear Range (CFU/gm)
Culture	**$10 - 10^2$**	**$10^2 - 10^9$**
RTD-PCR	**$10^3 - 10^4$**	**$10^6 - 10^8$**
PCR	**$10^4 - 10^5$**	**$10^5 - 10^7$**
ELISA-PCR	**$10^3 - 10^5$**	**$10^3 - 10^9$**

Pirnay JP et al. Crit Care (2000)4(4):255-61

Table 1. *Detection limits of various methods for detecting microorganisms. The units are colony forming units per gram stool (CFU/g).*

PCR (16S rDNA) is also useful for phylogenetic characterization ("community census") of very abundant microbial communities clustered into enterotypes that appear to be associated with health and specific diseases.

PCR-based platforms have significant limitations in the context of clinical medicine, especially for Complementary and Integrative medicine (CAM) in a non-acute care setting. CAM-oriented clinicians are interested in global changes in the composition and quantity of gastrointestinal microbiota, even in the absence of enteropathogens. That is because such perturbations can be the cause of changes in colonic metabolism that can be associated with significant symptoms of dysbiosis and various diseases. Culture is criticized for the limited number of microorganisms that can be grown compared to the vast number of microorganisms that have been detected using DNA methodologies. That criticism is used to argue that PCR is superior for evaluation of gastrointestinal microbiota. Culturing all gastrointestinal microorganisms is indeed a daunting if not impossible task. However, to put things in perspective, it should be noted that when MALDI-TOF MS is coupled with high complexity microbiology Doctor's Data has the capability to identify at least 1,240 bacteria and yeast organisms- that does not include speciation of the many *Lactobacilli* or *Bifidobacteria* species. Ironically, PCR requires a primer for identification of every genus (let alone species). Therefore, a reliable RTD-PCR based evaluation of microorganisms in a stool specimen that is even $1/10^{th}$ as comprehensive as that of the culture/MALDI-TOF MS approach would be absolutely cost prohibitive (if possible). Changes in the balance among the prevalent beneficial, expected and even commensal bacteria and their metabolic activities are as important as the absence of the relatively minute levels of pathogenic bacteria in many symptomatic patients. Some may recall Dr. Leo Galland's discussions of "deficiency dysbiosis." So, do you want to know as much as possible about the vast array of microorganisms that are present in your patients' stool specimens or do you want to simply inquire about just a handful of microorganisms? In essence PCR-based methods are better suited to address the question "is **it** there" rather than "what is there?" The DDI culture/proteonomic approach is analogous to casting a broad net versus using a fishing pole to obtain a census of the types of fish that inhabit a specific pond.

The issues of sensitivity and specificity also need to be brought into light in consideration of methods for evaluating gastrointestinal microorganisms. The detection limits (DL) of commonly used methods are presented in Table 1.

Despite common misconception culture can be 2 to 3 orders of magnitude more sensitive than even RTD-PCR. In fact culture can reveal the presence of just one CFU/g for an enteric pathogen when an enrichment broth is included. PCR-based methods can be less sensitive in part due to a "high noise to signal ratio" obtained when rDNA primers try to find mates among a sea of DNA from a fecal specimen. Further, clinical specificity with respect to microbial identification can be problematic because the probes used often cannot differentiate closely related microorganisms that have highly conserved sequences of 16S rDNA. PCR-based methods can also be associated with inferior clinical specificity due to use of inappropriate primers, and errors introduced during the amplification process.

Summary

Doctor's Data has optimized identification of gastrointestinal microorganisms from cultured stool specimens using an accurate and reliable proteomic technique (MALDI-TOF MS) that identifies bacteria and yeast by spectral analysis of microorganisms' predominant ribosomal proteins. Real-time PCR platforms, albeit less sensitive than culture, have a place in acute care situations of infectious diarrheal disease when time is of the essence. However the Real-time PCR approach does not currently lend itself to comprehensive analysis of the vast array of gastrointestinal microorganisms. Next- generation full genetic sequencing techniques in the research setting are moving forward and will further advance our understanding of the role of the gastrointestinal microbiome in health and disease. Currently, culture/MALDI-TOF MS technology is the most sensitive and discriminating way to identify the greatest number of beneficial/expected, commensal and pathogenic gastrointestinal microorganisms present in your patients.

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Evaluation of a Commercial DNA Stool Test

In 2007 a CLIA licensed diagnostic testing laboratory (Subject Laboratory) began offering a stool-screening test that uses a proprietary DNA method reported as "DNA-ELISA" (not Real-time Detection PCR) in effort to identify stool microbiota. Claims have been made that their DNA assessment is specific and accurate, and is more sensitive than traditional laboratory methods (e.g. culture). To test the accuracy and specificity of the proprietary DNA method, an external proficiency analysis study was conducted by a highly respected independent Life Sciences research institution (IIT Research Institute, Chicago, IL). The results of the study were presented at the 112th General Meeting of the American Society of Microbiology (Gingras BA, Duncan SB, Schuyeller NJ, Schreckenberger PC. Assessment of the Diagnostic Accuracy of Recently Introduced DNA Stool Screening Test. Abstr. 112th Gen. Mtg. Am. Soc. Microbiol., San Francisco, CA June 18, 2012).

Experimental Design and Methods

A human stool pool served as the consistent control matrix for all samples. The stool pool was tested extensively, using conventional methodologies, on two separate days and found to be free of entero pathogenic bacteria, yeast and parasites. Bacterial pathogens, authentic banked cell lines of *Shigella sonnei, Salmonella typhi, Escherichia coli 0157:H7, Campylobacter jejuni, Vibrio parahemolyticus, Aeromonas caviae, Plesiomonas shigelloides, Edwardsiella tarda, Yersinia enterocolitica, and Clostridium difficile,* were grown in culture and added in known concentrations to collection vials provided by the Subject Laboratory. Following the collection kit instructions, one gram of stool was added to each of three vials provided. The three vials contained either C&S Medium, 10% Formalin Fixative or a Nucleic Acid Collection Solution. Each vial was subsequently spiked with the bacterial preparations at final levels that are clinically significant, and at levels grossly higher in order to get into the Subject Laboratory's stated cutoff for clinically significant pathogenic bacteria (1 x 10^3 CFU/g). Their stated cutoff value for clinical significance is highly questionable because a patient would likely be very symptomatic if an organism such as *Campylobacter jejuni* was detected by culture at < 1 X 10^2 (100) CFU/g. Perhaps the Subject Laboratory is suggesting that their lowest level of *detection* is 1.0 x 10^3 (1,000) CFU/g for pathogenic bacteria. Final concentrations of spiked bacteria were determined in quintuplicate by culture. Thirty-four samples were shipped to the Subject Laboratory via overnight courier on the day of preparation, and the samples were also sent to reference laboratories for evaluation of entero pathogenic bacteria and parasites.

Results

Bacteria — Thirty-one samples each containing one enteric pathogen (at different concentrations) and 3 unspiked samples (controls) were tested. All 31 samples spiked with enteric pathogens were reported negative for pathogenic bacteria- 100% false negative. All 10 intestinal pathogens were recovered by reference laboratories using high complexity clinical microbiology techniques. One each of the 34 stool samples were reported to contain the commensal bacteria *Bacillus spp*. (10^7), *Staphylococcus aureus* (10^8) or *Klebsiella pneumonia* (10^7)- when all samples were from same stool pool. Complete results are presented in Table 1 (on page 7).

Parasites — Fifteen of 34 samples from the same stool pool (44%) were reported to contain No Ova or Parasites, and 17 of the 34 samples (50%) were reported as "**Parasite Present, taxonomy unavailable**." Three of 34 specimens were reported positive for *Cryptosporidium sp.* One sample that was spiked with *P. shigelloides* bacteria was reported negative for pathogenic bacteria but positive for *Enterobius vermicularis* (pin worm). All 34 samples contained the very same stool pool, and no parasites were detected by the reference laboratories using conventional O & P techniques and immunoassays for *Cryptosporidium* and *Giardia lamblia*.

An unanticipated finding was that the proprietary DNA methodology yielded random and non-specific results for parasites, and inconsistent results for yeast as well.

Yeast/ Fungi — Two of the 34 samples (5.9%) from the same stool pool were reported to contain yeast, one with *Candida spp.* (2+, ≥ 10^6 pg DNA/g) and one with *Geotricum* (4+, 10^3 pg DNA/g). Yeast were not detected by microbiological methods nor were any of the samples spiked with yeast.

Conclusion

The results of the proficiency study indicate that the proprietary DNA methodology employed by the Subject Laboratory was not able to detect pathogenic bacteria at levels that are known to be clinically significant, and grossly higher. An unanticipated finding was that the proprietary DNA methodology yielded random and non-specific results for parasites, and inconsistent results for yeast as well. Claims made by the Subject Laboratory that their DNA assessment of stool specimens is specific and accurate could not be supported.

Evaluation of a Commercial DNA Stool Test							
Sample ID	**Organism Added to Normal Stool Specimen**	**Quantity**	**Normal Stool Flora**	**Opportunistic Bacteria**	**Pathogenic Bacteria**	**Yeast/ Fungi**	**Parasites**
1	*Shigella sonnei*	$3.4x10^2$ CFU/g	+	-	-	-	Parasite Present; taxonomy unavailable
2	*Shigella sonnei*	$3.4x10^5$ CFU/g	+	-	-	-	Parasite Present; taxonomy unavailable
3	*Salmonella typhi*	$4.4x10^2$ CFU/g	+	-	-	-	Parasite Present; taxonomy unavailable
4	*Salmonella typhi*	$4.4x10^5$ CFU/g	+	-	-	4+ => 1000000pg DNA/g specimen *Geotricum* sp.	No Ova or Parasites
5	*E. coli* 0157:H7	$2.8x10^2$ CFU/g	+	-	-	-	*Cryptosporidium* sp. Positive, Parasite Present; taxonomy unavailable
6	*E. coli* 0157:H7	$2.8x10^5$ CFU/g	+	-	-	-	Parasite Present; taxonomy unavailable
7	*Campylobacter jejuni*	$2.8x10^2$ CFU/g	+	-	-	-	Parasite Present; taxonomy unavailable
8	*Campylobacter jejuni*	$2.8x10^5$ CFU/g	+	$7.3 X 10^7$ *Bacillus* sp.	-	-	No Ova or Parasites
9	*Vibrio parahemolyticus*	$5.8x10^1$ CFU/g	+	-	-	-	Parasite Present; taxonomy unavailable
10	*Vibrio parahemolyticus*	$5.8x10^4$ CFU/g	+	-	-	-	*Cryptosporidium* sp. Positive
11	*Aeromonas caviae*	$3.4x10^2$ CFU/g	+	-	-	-	Parasite Present; taxonomy unavailable
12	*Aeromonas caviae*	$3.4x10^5$ CFU/g	+	-	-	-	Parasite Present; taxonomy unavailable
13	*Plesiomonas shigelloides*	$4.4x10^2$ CFU/g	+	-	-	-	Parasite Present; taxonomy unavailable
14	*Plesiomonas shigelloides*	$4.4x10^5$ CFU/g	+	-	-	-	Enterobius vermicularis Positive
15	*Edwardsiella tarda*	$9.5x10^2$ CFU/g	+	-	-	-	*Cryptosporidium* sp. Positive, Parasite Present; taxonomy unavailable
16	*Edwardsiella tarda*	$2.4x10^3$ CFU/g	+	-	-	2+ => 1000pg DNA/g specimen *Candida* sp.	Parasite Present; taxonomy unavailable
17	*Edwardsiella tarda*	$9.5x10^5$ CFU/g	+	-	-	-	Parasite Present; taxonomy unavailable
18	*Yersinia enterocolitica*	$5.0x10^2$ CFU/g	+	$1.0 X 10^8$ *Staphylococcus aureus*	-	-	No Ova or Parasites
19	*Yersinia enterocolitica*	$5.0x10^5$ CFU/g	+	-	-	-	Parasite Present; taxonomy unavailable
20	*Clostridium difficile*	$2.4x10^1$ CFU/g	+	-	-	-	No Ova or Parasites
21	*Clostridium difficile*	$2.4x10^4$ CFU/g	+	-	-	-	No Ova or Parasites
22	Normal Stool Flora	N/A	+	-	-	-	No Ova or Parasites
23	Normal Stool Flora	N/A	+	-	-	-	No Ova or Parasites
24	*Shigella sonnei*	$6.5x10^3$ CFU/g	+	-	-	-	No Ova or Parasites
25	*Shigella sonnei*	$6.5x10^6$ CFU/g	+	-	-	-	No Ova or Parasites
26	*Yersinia enterocolitica*	$9.0x10^3$ CFU/g	+	-	-	-	No Ova or Parasites
27	*Yersinia enterocolitica*	$9.0x10^6$ CFU/g	+	-	-	-	No Ova or Parasites
28	*E. coli* 0157:H7	$5.6x10^3$ CFU/g	+	-	-	-	Parasite Present; taxonomy unavailable
29	*E. coli* 0157:H7	$5.6x10^6$ CFU/g	+	-	-	-	No Ova or Parasites
30	*Vibrio parahemolyticus*	$9.2x10^2$ CFU/g	+	-	-	-	Parasite Present; taxonomy unavailable
31	*Vibrio parahemolyticus*	$9.2x10^5$ CFU/g	+	$6.1 X 10^7$ *Klebsiella pneumoniae*	-	-	Parasite Present; taxonomy unavailable
32	*Clostridium difficile*	$5.4x10^2$ CFU/g	+	-	-	-	No Ova or Parasites
33	*Clostridium difficile*	$5.4x10^5$ CFU/g	+	-	-	-	No Ova or Parasites
34	Normal Stool Flora	N/A	+	-	-	-	No Ova or Parasites
** (-) indicates bacteria were not present; (+) indicates bacteria were present							

Table 1. *Results of stool analysis conducted by the Subject Laboratory.*

Extremely Comprehensive Stool Testing – More than pathogen detection

Andrea Gruszecki, ND

We live in interesting times. New technologies allow for laboratory identification of microbial species that were previously unavailable or unidentifiable. With the advent of newer technologies, where does that leave the old, reliable laboratory standards? Right on center stage, where they belong. Current DNA technologies, using validated platforms (now used for rapid identification in major medical institutions) may identify known pathogens but cannot provide information about antibiotic sensitivities. A gene present in the microbiome is not necessarily actively expressed. Nor do advances in microbial identification provide information about a patient's digestive status, immune status, tissue inflammation, etc. Many types of information are required to properly assess a symptomatic patient, and pathogen detection is only one piece of a much larger puzzle.

How can the results of a Comprehensive Stool Analysis be used clinically?

Clinical success requires more than treating symptoms. Comprehensive assessments and diagnoses will enable targeted treatment of the cause of symptoms. Many patients present with gastrointestinal complaints. Many of these complaints will be *functional* (the result of abnormal motility, digestion, sensation or brain-gut-microbiome dysregulation); some may be associated with an undiagnosed food allergy or intolerance, such as Celiac disease. Some of these patients will have an undiagnosed disease process such as Inflammatory Bowel Disease (IBD). Blind treatment of symptoms rarely creates health or long-term improvement in these patients; a missed diagnosis may be truly harmful.

What are the elements of a Comprehensive Stool Analysis?

A Comprehensive Stool Analysis (CSA) will include information to evaluate the composition of the gut flora, and isolate any pathogens, dysbiotic species or yeasts present. The analysis will provide biomarkers for digestion and absorption, inflammation, and immune status. A functional assessment of microbiome functions is provided by measurement of short chain fatty acid (SCFA) production. Biomarkers of overall health include testing for stool pH, red blood cells, white blood cells, mucus and occult blood in the stool. All of these parameters are evaluated and a visual inspection of the stool is performed to assess color and consistency.

When might a Comprehensive Stool Analysis be ordered?

A CSA should be considered for any patient with gastrointestinal symptoms. Other complaints that may be associated with gastrointestinal dysfunctions include skin conditions, arthritis, carbohydrate sensitivity, foul breath, attention deficit disorders, autistic behaviors or symptoms of poor detoxification.

Microbiology

Culture and sensitivity testing remains the international standard in microbiology. With proper collection and handling, anaerobes, beneficial bacteria, commensal bacteria and pathogens are easily recovered in the laboratory. Identifying and reporting the greatest number of different bacteria present is clinically important; certain species reported by DDI, including *Bacteroides, Enterococcus* and *Clostridia* species and their metabolites, can translocate from the gut into the circulation and cause symptoms when they colonize other tissues in the body. DDI often detects anaerobic pathogens, such as *Clostridia difficile*, during routine microbiology. When *C. difficile* is identified, "reflex" testing for toxins A and B is performed using a very sensitive and specific DNA analysis.

While the most recently developed genetic technologies are gaining in precision and accuracy, the rate of false positives and negatives from PCR testing, when compared side by side with conventional culture may still be unacceptably high, as broad-range 16s-rDNA probes may not be suited for all applications. Standardized probes have only been developed for common virulent pathogens and ultra pure probes needed to identify most beneficial or commensal gut bacteria down to the species level have not been standardized or validated. Samples used for PCR testing cannot be cultured to provide microbial sensitivities to guide treatment with pharmaceuticals or natural anti-microbials. Most major medical institutions continue to use culture and sensitivities to confirm their PCR-based rapid testing. Conventional culture is able to identify most organisms isolated down to species level, and excels at identifying species that have a great deal of genetic similarity near their 16S sites and species with thick cell walls. Such species may be missed or misidentified by some PCR technologies' probes. Doctor's Data uses MALDI-TOF MS for the most accurate identification of species and even sub-species of bacteria and yeast.

Parasitology

Slide microscopy and immunoassay testing continue to be valuable components of parasitology. All major intestinal helminth (worm) infections are diagnosed under the microscope, and may be for the foreseeable future. The advent of newer technologies has improved parasite identification. MALDI-TOF is able to identify proteins specific to parasites. The blending of new and proven technologies will improve the sensitivity of parasite detection.

Digestion and Absorption Biomarkers

Fecal Elastase is a measure of pancreatic exocrine function, and more generally, it serves as an indicator for the presence of sufficient digestive enzyme secretion. The presence of muscle or vegetable fibers in stool may indicate mechanical issues (mastication) or incomplete digestion. The fat stain is a useful biomarker that may indicate a specific need for supplemental lipase. A positive fat stain may also serve as a "red flag"; fats may not be properly digested in cases of gluten intolerance, food sensitivity, or abnormally rapid transit time. Carbohydrates present in the stool sample may indicate excessive consumption of simple carbohydrates, lack of carbohydrate

specific enzymes, or gluten enteropathy. The use of specific digestive enzymes, elimination of offending foods, and modification of eating habits are therapies used to improve digestion.

Inflammation Biomarkers

Inflammation is an integral part of disease processes that may be associated with Irritable Bowel Disease (IBD), gluten enteropathy, infections, or pharmaceuticals. Lysozyme is a general marker of inflammation. It may be elevated from a variety of causes. Lactoferrin is an FDA-cleared *specific* biomarker for Inflammatory Bowel Disease; it may be used to monitor therapies and remissions in IBD. Lactoferrin has been shown to be equivalent to calprotectin in detecting IBD in comparison studies. White blood cells may occur with inflammation or infection. Mucus may also be present with inflammation, digestive disorders, infections, pelvic abscess or proctitis. Avoidance of inflammatory triggers and use of anti-inflammatory therapies may both be considered to decrease inflammation.

Immunology

The gastrointestinal mucosal immune system has the difficult task of mounting protective responses to invading microorganisms while allowing the presence of beneficial bacteria and foods. Secretory IgA (sIgA) is secreted by the gut mucosa and is an important component of the immune barrier. An elevated sIgA indicates an ongoing or acute immune challenge. A diminished level of sIgA may indicate either a chronic challenge that depletes sIgA or an inability to manufacture sIgA (primary sIgA deficiency). Levels of sIgA may be improved with probiotics, *Saccharomyces boulardii*, zinc, vitamins A and D, and glutamine.

Short Chain Fatty Acids

Short chain fatty acids (SCFAs) are important biomarkers and protectors of colon health. The production of SCFA's depends on the gut microbes present in the GI tract, diet, and transit time. The presence of specific beneficial bacteria *(Bifidobacterium* and *Lactobacillus spp.)* and SCFAs in the colon may improve cellular health and moderate cancer risks. SCFAs modulate colon pH influence microbial populations and ammonia levels; they may indirectly influence mineral absorption. The primary SCFAs produced in the colon include:

- Acetate – metabolized by peripheral tissues
- Propionate – used primarily by the liver
- Butyrate - primary fuel source for colonocytes
  - modulates pH and secondary bile acid formation
  - modulates serotonin release
  - may have anti-inflammatory and anti-cancer properties
  - enhances the anti-*Candida* activity of macrophages and, azole anti-fungals

SCFA's that are not used locally pass into the circulation and are metabolized by the liver or muscle cells. High levels of SCFAs may indicate simple carbohydrate malabsorption, decreased transit times or dysbiosis. Low levels usually indicate low populations of beneficial bacteria and/or insufficient intake of soluble fiber.

Intestinal Health Markers

The presence of red blood cells (RBCs) may confirm infection or inflammation indicated by other biomarkers of the CSA. RBCs can also indicate the presence of local problems such as diverticulitis, hemorrhoids or anal fistula; such conditions may coat the exterior of a formed stool with fresh blood. The occult blood test indicates the presence of hemoglobin released from RBCs elsewhere in the GI tract, and, if occult blood is positive over time (4-6 weeks and two retests), the patient should be further evaluated to find the source of the occult upper GI bleeding. The presence of RBCs and/or occult blood may indicate serious problems such as colon or gastrointestinal cancers. These tests guide providers to identify and treat sources of bleeding in the GI tract.

Stool pH will vary based on several factors, including diet, the abundance of beneficial bacteria, and bowel transit time. Stool pH generally decreases with increased dietary fiber or a rapid transit time; the latter may be associated with poor nutrient absorption. Stool pH generally increases with increased transit time or constipation.

Macroscopic Appearances

Stool color and consistency may provide information that leads to further assessment and treatment. Stool consistency reflects the water content and transit time of the stool. Stool color may indicate various clinical disorders, once dietary color inputs (beets, greens, food colorings, etc.) have been accounted for. Green stool may indicate a lack of bile metabolism in the gut lumen. Clay-white stools indicate insufficient bile secretion. A foul, yellow, greasy stool indicates either excess fat in the diet or insufficient fat digestion, which may be associated with gluten intolerance. Black tarry stools indicate bleeding from the upper GI tract and will correlate with a positive occult blood test. Bright red in the stool indicates local blood from the colon or rectum, perhaps from hemorrhoids.

The Comprehensive Stool Analysis is a great tool for clinicians who are interested in truly comprehending a patient's GI health and functions. New technologies, combined with existing, reliable, standardized laboratory tests, provide a wealth of information about the microbiome, pathogens, parasites, digestion, inflammation, and gut health.

CONTINUED ON PAGE 10

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BEHIND THE SCENES AT DDI

Meet the Microbiology Team

The Microbiology Team at Doctor's Data, under the Direction of world-renowned Microbiologist Dr. Paul Schreckenberger and the Management of Hope Beilfuss, possesses a unique skillset that has been carefully cultivated to meet the growing demands of a surging segment of Laboratory Medicine.

Dr. Schreckenberger is a Diplomate of the American Board of Medical Microbiology, D(ABMM), and a Fellow in the American Academy of Microbiology, F(AAM), and is co-author of *Koneman's Color Atlas and Textbook of Diagnostic Microbiology*. He has written over 250 articles, abstracts, monographs and self-study courses and serves as a member of the editorial boards for the Journal of Clinical Microbiology and Diagnostic Microbiology and Infectious Diseases. He is an active participant in the Clinical and Laboratory Standards Institute (CLSI) Subcommittee on Antimicrobial Susceptibility Testing, serving on a number of Working Groups. Dr. Schreckenberger has lectured extensively both nationally and internationally, presenting both workshops and seminars on a variety of topics in clinical microbiology including intestinal dysbiosis and the gut microbiome.

Hope Beilfuss joined Doctor's Data in 2008 as the Microbiology Manager, following an extensive tenure early in her career at Loyola University Medical Center as a specialist in Chromatography, years of experience in microbiology and parasitology at the Brookfield Zoo, and experience in genome sequencing and hospital informatics at Edward Hospital in Naperville. This distinctive combination of experience and expertise lends itself directly to the specialty offerings of the Microbiology Department at Doctor's Data. Hope explains that it is very rare to find this menu of offerings in a laboratory that is not a national reference laboratory or part of an academic medical center.

Throughout the 80's and 90's, the emphasis in Microbiology was to assure that pathogenic organisms were being identified so that proper treatment was prescribed. As Hope explains, "Historically, only the pathogens that emerged from a complex culture were identified and tested for resistance or susceptibility against commonly prescribed antibiotics. Now, the emphasis has become identifying every microbe present in the sample, and testing the pathogenic organisms against a panel of botanical as well as prescriptive antimicrobials." Recently, the emphasis has shifted to looking not only at rapid and accurate identification of pathogens and providing information on antibiotic treatments, but also to provide an assurance that the normal flora and the microbiota are in appropriate balance. Doing so requires a far more comprehensive approach to isolation and identification that few laboratories are able to meet. In 2012, Doctor's Data supplemented its already extensive comprehensive test menu, and acquired Matrix Assisted Laser Desorption Ionization - Time of Flight (MALDI-TOF) Mass Spectrophotometric equipment to facilitate the timely and accurate identification of actively growing yeast isolates, and aerobic and anaerobic bacteria. Providing clinicians with information on the presence, absence, or balance of organisms, whether commensal or pathogenic, allows for the assessment of the gut microbiota and directed treatments for patients determined to have intestinal dysbiosis leading ultimately to better patient outcomes.

DDI Microbiology department with over 200 years of experience pictured left to right.

BACK ROW: Semsa Memic, Jean Brankin, Luz Jimenez, Swati Rana, Gloria Martinez, LaToya Hayden, Syeda Razvi

FRONT ROW: Lori Engelhardt, Hope Beilfuss, Dr. Paul Schreckenberger, Angela Kopenec, Kayla Colwell

Angela Kopenec, Microbiology Supervisor, explains, "Our staff truly feels like a family. We depend on one another, and learn from each other. We have a wonderful combination of personalities and skills." Prior to coming to Doctor's Data, Angela, a certified Medical Technologist, worked as a microbiologist at the Hospital of the University of Pennsylvania while completing her Master's Degree in Health Administration. She is also an experienced Infection Control Practitioner.

Both Hope and Angela are quick to highlight the many years of experience of the medical technologists and laboratory technicians in Microbiology, who average more than 10 years of clinical laboratory experience, with expertise in mycology, parasitology, and virology, in addition to general and anaerobic bacteriology.

Doctor's Data embraces cutting edge technologies, and continues to demonstrate its confidence in the Microbiology Staff by further expanding its offerings to include bacterial genomic sequencing to complement the MALDI-TOF identification capabilities. Look for these offerings to be available later in 2013.

KNOWLEDGE SPOT

Why did DDI choose MALDI-TOF mass spectroscopy instead of a PCR-based technology?

***M**atrix-**A**ssisted-**L**aser-**D**esorption/**I**onization **T**ime **O**f **F**light (MALDI-TOF) identifies bacteria and yeast through their unique signature protein patterns (see "Proteomics: A Revolution in the Evaluation of Gastrointestinal Microflora" by Dr. Quig in this newsletter). The MALDI-TOF technology for the identification of microbes is ranked third in the "Top Ten" of Cleveland Clinic's 2013 list of Medical Innovations expected to improve patient care in the next year. MALDI-TOF combines the greater sensitivity of microbial culture with the speed and superior accuracy of protein-pattern recognition.*

Some research labs have developed real-time PCR methods to identify a small panel of diarrhea-associated bacteria from the gastro-intestinal tract. As they are less sensitive, the real-time results are always confirmed with culture and sensitivities, nor are they commercially available. Thus far, commercial claims regarding the accuracy of proprietary PCR-ELISA assessments of microbes and pathogens in stool remain unsupported by independent scientific investigation. (Gingras, B.A.; Duncan, S.B.; Schueller, N.J. and Shreckenberger, P.C. 2012)

MALDI-TOF MS, combined with DDI's current microbiology assessments provides a new level of detail about the bacteria and yeast of the gastrointestinal microbiome, while preserving the ability to provide direct susceptibility testing.

Gingras BA, Duncan SB, Schueller NJ, Schreckenberger PC. Assessment of the Diagnostic Accuracy of Recently Introduced DNA Stool Screening Test. Abstr. 112th Gen. Mtg. Am. Soc. Microbiol., San Francisco, CA June 18, 2012.

Why are there two inflammatory markers on the Comprehensive Stool Analysis? What is the difference between Lysozyme and Lactoferrin?

Results from the two markers help clinicians differentiate IBD from other less severe inflammatory processes in the gut.

Lysozyme is a general inflammatory marker that is released from intestinal granulocytes such as neutrophils, basophils or eosiniphils. Lysozyme may be elevated in response to pathogens, parasites, allergens or autoimmune reactions. Lysozyme may also be elevated in Crohn's Disease, but it is not a specific marker for Crohn's or other Inflammatory Bowel Disease (IBD).

Lactoferrin is an FDA cleared in vitro diagnostic test; this inflammatory marker is specific for IBD, it helps differentiate IBD from other gastrointestinal disorders.

Is there any truth to the rumor that the stool transport media can't support strict anaerobes or other gastrointestinal (GI) bacteria?

The Cary-Blair transport media used by DDI is specifically designed with low levels of nutrients to suspend the growth of stool bacteria during transport, and inhibit the growth of other bacteria. The media is buffered to prevent shifts in pH during transport, and contains a reducing agent designed to markedly decrease oxygen levels to favor the facultative and strict anaerobic species known to inhabit the GI tract. When properly collected and shipped, beneficial and pathogenic species may be successfully recovered quantitatively from the transport media for up to 14 days. Once the bacteria and yeast have been cultured, susceptibility testing to pharmaceutical agents and botanical or natural agents is completed, to optimize clinical treatment decisions and improve patient outcomes.

For a Parasitology x3 test, does DDI check all three stool samples for parasites? Other labs pool the specimens and check only once. What it the difference and which is better?

A recent 2006 study by Massachusetts General Hospital explored this very question. The study found that a single pooled sample was adequate only when there were high rates of infection in the population. In addition, the study found that a review of additional slides, once a parasite was identified in a sample, identified a second *parasite not appreciated in the original slide, ten percent of the time. Doctor's Data examines a slide for each separate stool sample submitted, and reviews all three slides once a parasite is identified, to identify that second 1-in-10 second parasite and optimize clinical outcomes.*

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