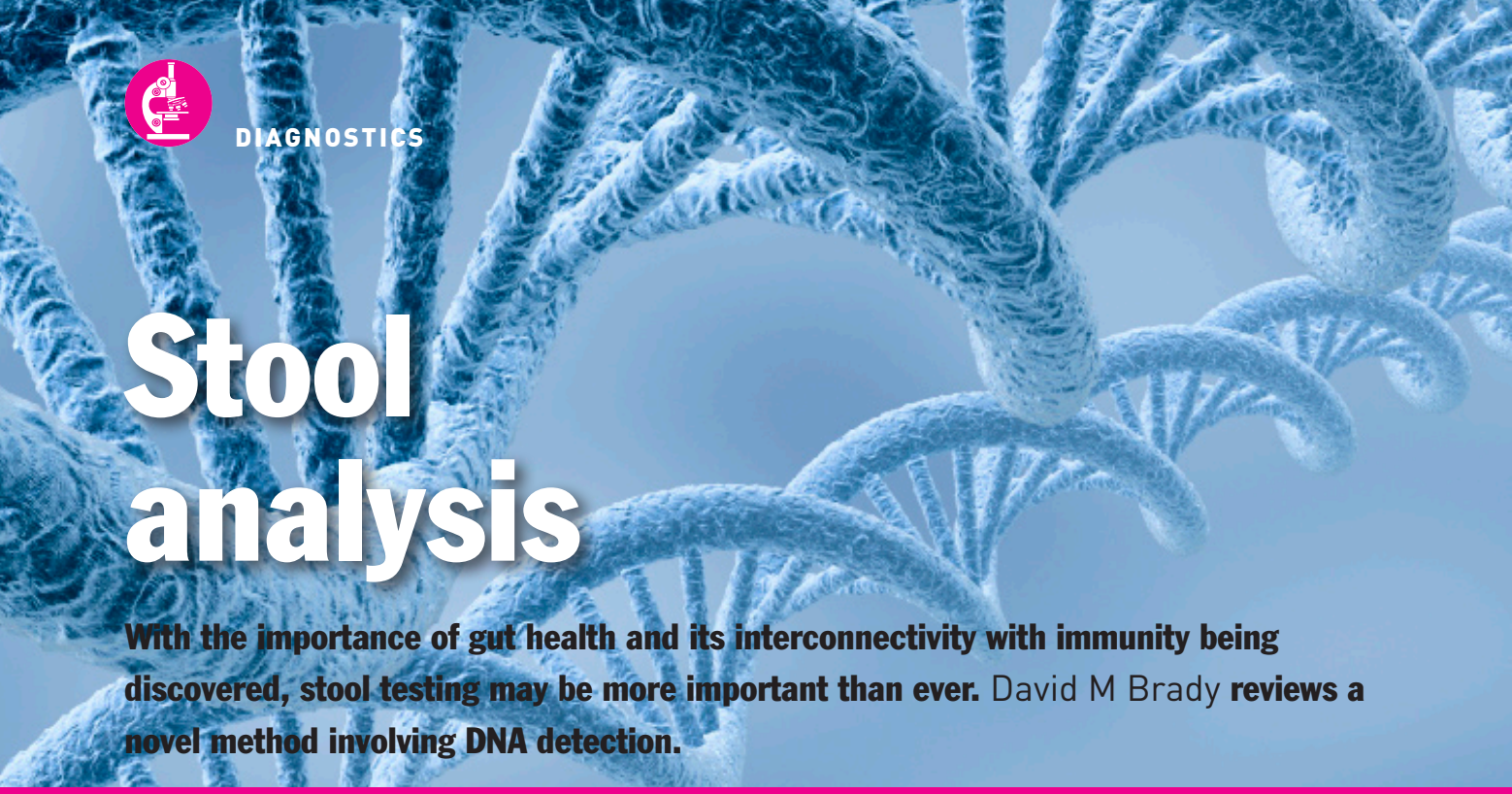

DIAGNOSTICS

Stool analysis

With the importance of gut health and its interconnectivity with immunity being discovered, stool testing may be more important than ever. David M Brady **reviews a novel method involving DNA detection.**

This article is written by an independent expert in his field and reviewed for its value to GPs. The *JCM* appreciates the support of Diagnostic Insight in providing quality information for the benefit of its readers.

David M Brady, ND, DC, CCN, DACBN, is Director, Human Nutrition Institute, and Associate Professor of Clinical Sciences, University of Bridgeport, Connecticut, US, and an independent consultant to Metametrix

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SUMMARY

- Excessive colonisation of the gut by undesirable microorganisms can alter the metabolic or immunologic status of the host and can cause or complicate many symptoms and conditions
- A Bacteroidetes species decrease relative to Firmicutes in the gut has been associated with accumulation of body fat and insulin resistance in both humans and experimental animals
- 98% of faecal bacteria are anaerobic, making standard faecal culture techniques not an ideal method to report amounts of total bacteria
- DNA analysis eliminates the problem of transporting microbes growth by killing all organisms and capturing the exact balance present at collection
- PCR amplification of target microbial DNA is a reliable and sensitive method for organism detection
- Due to ~100% sensitivity and specificity of DNA analysis combined with low amounts of genomic DNA needed, only one faecal sample is needed for detecting parasites

The intestinal flora is a complex ecosystem consisting of over 400 bacterial species that greatly outnumber the total number of cells making up the entire human body.[1] These metabolically active bacteria reside close to the absorptive mucosal surface and are capable of a remarkable repertoire of transforming chemical reactions. Any orally taken compound, or a compound entering the intestine through the biliary tract by secretion directly into the lumen, is a potential substrate for bacterial transformation.

Anaerobic bacteria are the predominant microorganisms in the human GI tract, outnumbering aerobes by a factor of 10,000 to one. The most abundant and beneficial or benign anaerobes are *Bifidobacterium, Bacteroides, Fusobacterium, Clostridium, Eubacterium, Peptococcus* and *Peptostreptococcus* sp. *Bifidobacterium* can comprise up to 25% of the total flora in a healthy adult. A great many other species are present in lesser numbers.[2] An imbalance in proportion and numbers of these species can be induced by broad-spectrum antibiotic use. This leads to the dominance of other bacterial species, including *Pseudomonas, Enterobacter, Serratia, Klebsiella, Citrobacter, Proteus, Providencia* and fungi, especially yeasts such as *Candida*. In health, the upper GI tract is sparsely populated with microorganisms. Gram-positive, facultative forms such as *Streptococcus, Staphylococcus* and *Lactobacillus* typically survive gastric secretions and bile acids.[3] In the distal ileum, concentrations of bacteria increase and the Gram-negative bacteria such as *Bacteroides,*

Bifidobacterium, Fusobacterium and *Clostridium* outnumber the Gram-positive. Beyond the ileocecal valve, the bacterial concentration increases steeply to 10^{12} colony-forming units (CFUs) per milliliter of faecal material. By the time they are passed as stools, the large majority of the bacteria are no longer viable.

Excessive colonisation of the gut by undesirable microorganisms alters the metabolic or immunologic status of the host.[4,5] When this state leads to disease or dysfunction, it has been termed 'dysbiosis' to distinguish it from the correct balance denoted as orthobiosis.[6] Symptoms and conditions thought to be caused or complicated by dysbiosis include inflammatory bowel diseases, inflammatory or autoimmune disorders, food allergy, atopic eczema, unexplained fatigue, arthritis, mental/emotional disorders in children and adults, malnutrition and breast and colon cancer.

Intestinal microbiota associated with obesity

There are two main bacterial groups in the human GI tract, Bacteroidetes and Firmicutes. Recent research has associated an imbalance in these groups with obesity and insulin resistance (IR). The Firmicutes class of bacteria includes *Bacillus, Clostridium, Lactobacillus, Streptomyces* and *Mycoplasma* and is very efficient at metabolising plant polysaccharides into monosaccharides and short-chain fatty acids. These can be absorbed by the gut and converted to more complex lipids in the liver. In addition, this group secretes a compound that results in increased activity of lipoprotein lipase in adipocytes, resulting in enhanced storage of these lipids.[11] The Bacteroidetes group, which includes *Bacteroides* and *Prevotella* sp., are not as efficient in this function. A Bacteroidetes species decrease relative to Firmicutes in the gut has been associated with significant accumulation of body fat both in humans and in experimental animals.[12,13] When germ-free mice are inoculated with this imbalance of microbiota, they have significantly greater accumulations of total body fat and increased IR. Similar animals with the better balance remain lean, even though they have identical diets.[11] The authors also found that an imbalanced microbial population decreases AMP-activated protein kinase activity, thereby reducing energy available for muscular activity.[14] In humans, lean individuals have a higher percentage of Bacteroidetes relative to Firmicutes than do obese individuals.[13] Interestingly, if obese humans are put on low-carbohydrate or low-fat diets and lose weight, their microbial balance also improves.

Additionally, in mice with diabetes induced by a high-fat diet, *Bifidobacter* sp. supplementation significantly improved glucose tolerance, glucose-induced insulin secretion and decreased endotoxaemia and some inflammatory cytokines.[15] In another study, improvements in IR and oral glucose tolerance, and decrease in plasma lipopolysaccharide concentrations related to endotoxaemia, were seen in mice treated with antibiotics.[16] These studies are indicating that the balance of these microbes in the gut is a major contributor to IR and that this can be remedied by improving that balance.

While obesity ultimately is caused by excess caloric intake, differences in gut microbial ecology may be an important component of energy homeostasis, metabolic status and inflammatory modulation. The use of specific diets or pre- and probiotic therapies may be able to significantly affect microbial balances that affect fat storage and IR. The ability to assess the balance of these 'fat bugs' in humans will potentially be an important advance in contributing to the resolution of a significant public health issue, namely obesity.

Difficulties in assessing microbiota content

Most studies of microbiota in the GI tract have used faecal samples. These do not necessarily represent the populations along the entire GI tract from stomach to rectum. Conditions and species can alter greatly along this tract and generally run from lower to higher population densities. Faecal samples most appropriately represent organisms growing in the colon. In addition, >98% of faecal bacteria will not grow in the presence of oxygen. Therefore, standard faecal culture techniques miss the majority of organisms present.

Conventional bacteriological methods such as microscopy, culture and identification are used for the analysis and/or quantification of the intestinal microbiota.[17–19] Limitations of conventional methods are their low sensitivities[20], inability to detect non-cultivatable bacteria and unknown species, time-consuming aspects, and low levels of reproducibility due to the multitude of species to be identified and quantified. In addition, the large differences in growth rates and requirements of the different species present in the human gut indicate that quantification by culture is bound to be inaccurate. These techniques have low sensitivities and are laborious and technically demanding. To overcome the problems of culture, techniques based on 16S ribosomal DNA (rDNA) genes were developed.[21,22]

Another problem with present stool analysis procedures is that of transport. Since analysis is culture dependent, sample collection must be done using nutrient broth containers to maintain microbial viability. This allows continued growth of species during transport until the sample is actually plated out for culture. This growth allows for a significant change in the balance of microbes present, since some species will more actively grow at the expense of others. DNA analysis circumvents this by placing the specimen in formalin vials for transport. This immediately kills all organisms, freezing the exact balance present at the time of collection.

Preliminary studies performed by Bralley et al (unpublished) show a significant decrease in faecal *Bifidobacter* sp. from collection to three days in the nutrient broth mixture. In contrast, *Staphylococcus aureus* had a fivefold increase in CFUs/gram of faecal matter in one day in the nutrient broth. *Candida* sp. also grew exponentially in the nutrient broth, and peaked on day 2. The stool samples fixed in formalin provided consistent readings from collection to day 3.

Since DNA hybridisation techniques detect only the genes of the microbiota, living specimens are not necessary. This allows

the clinician to develop the most appropriate therapy based on the patient's true gut microbiota, resulting in better clinical results.

Polymerase chain reaction (PCR)

One of the most important contributions to molecular biology is the advent of the polymerase chain reaction (PCR). PCR, a DNA and RNA-based technology, can detect a unique DNA sequence of an infectious agent in any body fluid, enabling fast and accurate identification. PCR does not depend on the ability of an organism to grow in culture. Furthermore, PCR is fast, sensitive and capable of copying a single DNA sequence of a viable or non-viable cell over a billion times within 3–5 hours. In addition, PCR methodology requires only 1–5 cells for detection, whereas a positive culture requires an inoculum equivalent to about 1000–5000 cells, making PCR the most sensitive detection method available (approximately 1000 times more sensitive).[23]

Advantages of PCR amplifications of target microbial DNA for organism detection over traditional culture techniques are many:

- ability to detect nonviable organisms that are not retrievable by culture-based methods
- ability to detect and identify organisms that cannot be cultured or are extremely difficult to grow (e.g. anaerobes)
- more rapid detection and identification of organisms that grow slowly (e.g. mycobacterium and fungi)

Stool testing comparison chart

	PCR-DNA stool analysis	Culture stool analysis
DNA identification of microbiota	YES	NO
One sample collection per patient (even for parasites)	YES	
Detects parasites in the smallest concentration per specimen	5 cells per gram	25,000 cells per gram
Detects the presence of drug-resistance genes	YES	NO
Identifies all of the targeted microbiota, including anaerobic organisms	100%	5%
Evaluates balance of microbes shown to contribute to weight gain	YES	NO
Multiple antibiotic and botanical sensitivities	YES	YES
Gliadin-specific sIgA and total sIgA	YES	?

CASE STUDY

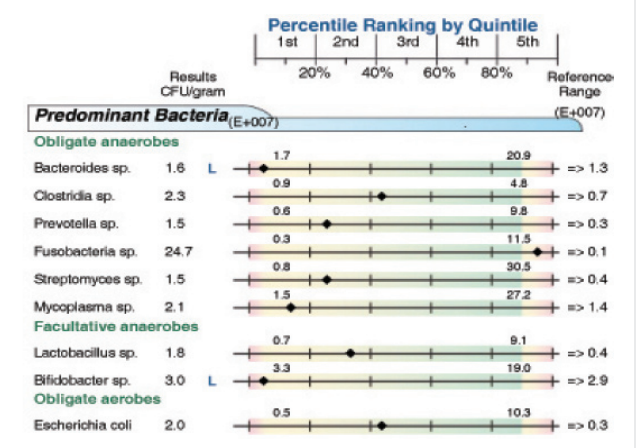
The problem I have experienced with stool tests has been the high rate of false negative results. I have often received results that show normal findings and are negative for flora issues or infections despite strong clinical suspicion for dysbiosis. When I have treated for infections despite a clear stool test, I have still had the expected good clinical result.

However, I have found PCR-derived stool reports now demonstrate a higher yield of infection positives for normal flora issues, opportunistic or pathogenic bacteria, yeast/fungal organisms and parasites. The case described below demonstrates this.

Irritable bowel

A 65-year-old man presented with a five-year history of IBS involving significant nausea on waking, chronic abdominal discomfort and post-prandial bloating. There was a history of dysentery during travel through South America in 1996 and Asia in 1998. Symptoms post-dated a case of Q Fever (treated with long-term tetracyclines) five years ago.

The GI Profile revealed



significant reduction in normal flora aerobes and anaerobes. While the majority of beneficial flora are low, *Fusobacteria* sp. is relatively high (24.7 colony-forming units/g). This is, by definition, dysbiosis and is further compounded by findings of the short-chain fatty acid (SCFA) tests. The imbalance in production of SCFA will continue to increase dysbiosis without intervention.

I prescribed a course of

- ability to detect previously unknown organism directly in clinical specimens by using broad-range DNA primers
- ability to quantitate infectious organisms' burden in patient specimens for better clinical responsiveness.

Parasitology

Parasitology is yet another field of microbiology to be greatly improved with genetic molecular technologies. Classically, parasites have been identified by microscopy and enzyme immunoassays.[24] In recent studies, molecular techniques have proven to be more sensitive and specific than classic laboratory methods.[24-26] Because Giardia cysts are shed sporadically and the number may vary from day to day, laboratories have adopted multiple stool collections to help increase identification rates for all parasite examinations.[26] And, even with the advent of antigen detection systems, there has long been uncertainty in diagnosis when no ova or parasites are found. Due to the nearly 100% sensitivity and specificity of DNA analysis and the need for very low amounts of genomic DNA (as low as 2.5 cells per gram)[26], multiple specimen collections and technically challenging microscopic identification have been alleviated. With PCR technology, only one faecal sample is needed for 100% sensitivity and specificity in parasitology examinations.

Conclusion

DNA analysis technology allows for a significant advancement in understanding of how GI tract microbiota affects human health. It improves patient care by giving clinicians greater options and more tools in treating patients. The increased speed of analysis and improved accuracy makes this a preferred method of stool analysis.

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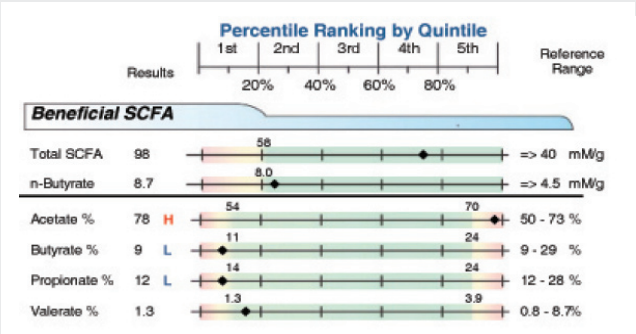

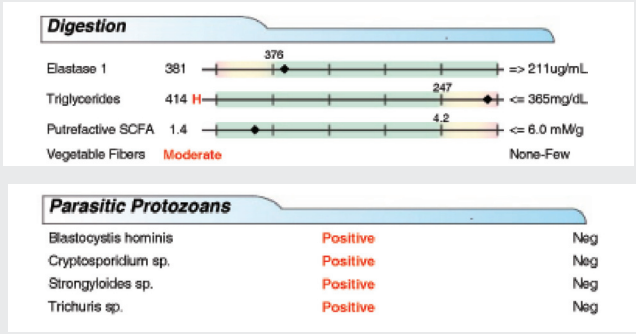

Zentel at 200mg bd for six days and this was repeated after one month. Over the following two months, he regained a healthy appetite, 3kg weight and some improvements in behaviour. Repeat testing has not yet been performed.

Elastase levels were low (381mcg/mL), indicating poor digestive enzyme production. High faecal triglycerides (414mg/dL) and total fats indicated malabsorption.

There was also infection with *B. hominis*, *Cryptosporidium, Strongyloides*, and *Trichuris ovis* (whipworm). Trichuris is a more common tropics infection in children, following swallowing of faeces-contaminated soil (or foodstuffs).

Initial treatment consisted of a dose of ivermectin for the *Strongyloides*, followed by a course of merbendazole (Vermox) bd for 3 days for the *Trichuris*.

Following these treatments, he reported a loss of his waking nausea and a dramatic improvement in his bloating and abdominal discomfort. The second phase of treatment involved commencing digestive enzymes, probiotics (VSL#3; Orphan Australia), a colostrum powder and a prebiotic.

Emmanuel Varipatis, MBBS, is an integrative GP