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# DNA DETECTION OF GUT MICROBIOTA

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## **Introduction**

The population of the microbiota of the human gastrointestinal (GI) tract is widely diverse and complex with a high population density. All major groups of organisms are represented. While predominately bacteria, a variety of protozoa are also present. In the colon there are over 10<sup>11</sup> bacterial cells per gram and over 400 different species. These bacterial cells outnumber host cells by at least a factor of 10. This microbial population has important influences on host physiological, nutritional and immunological processes. In fact, this biomass should more rightly be considered a rapidly adapting, renewable organ with considerable metabolic activity and significant influence on human health. Consequently there is renewed and growing interest in identifying the types and activities of these gut microbes.<sup>1</sup>

The normal, healthy balance in microbiota provides colonization resistance to pathogens. Since anaerobes comprise over 95% of these organisms, their analysis is of prime importance. Gut microbes might also stimulate immune responses to prevent conditions such as intestinal dysbiosis. Intestinal dysbiosis may be defined as a state of disordered microbial ecology that causes disease. Specifically, the concept of dysbiosis rests on the assumption that patterns of intestinal flora, specifically overgrowth of some microorganisms found commonly in intestinal flora, have an impact on human health. 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 both children and adults, malnutrition and breast and colon cancer.<sup>2,3</sup>

## **Difficulties in accurately assessing microbiota content**

Most studies of microbiota in the GI tract have used fecal 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. The stomach and proximal small intestine with highly acid conditions and rapid flow contain 10<sup>3</sup> to 10<sup>5</sup> bacteria per gram or ml of content. These are predominated by acid tolerant lactobacilli and streptococci bacteria. The distal small intestine to the ileocecal valve usually ranges to 10<sup>8</sup> bacteria per gram or ml of content. The large intestine generates the highest growth due to longer residence time and ranges from 10<sup>10</sup> to 10<sup>11</sup> bacteria per gram or ml of content. This region generates a low redox potential and high amount of short chain fatty acids.

Not only does the microbiota content change throughout the length of the GI tract, but there are also different microenvironments where these organisms can grow. At least four microhabi-

tats exist: the intestinal lumen, the unstirred mucus layer that covers the epithelium, the deeper mucus layer in the crypts between villi, and the surface mucosa of the epithelial cells.<sup>4,5</sup> Given this diverse ecological community, the question arises as to how to sample the various environments to identify populations of microbes and ultimately understand the host-microbe interactions. This problem is an extremely difficult one since any intervention to obtain a sample potentially disrupts the population. Fecal sampling has been used for years in microbiota assessment. But it should be understood that this sample primarily most appropriately represents organisms growing in the colon. In addition, >98% of fecal bacteria will not grow in oxygen.<sup>4</sup> Therefore, standard culture techniques miss the majority of organisms present.

## **Conventional Techniques versus New Technologies**

Conventional bacteriological methods like microscopy, culture, and identification are used for the analysis and/or quantification of the intestinal microbiota.<sup>6-8</sup> Limitations of conventional methods are their low sensitivities,<sup>9</sup> their inability to detect noncultivable bacteria and unknown species, their time-consuming aspects, and their low levels of reproducibility due to the multitude of species to be identified and quantified. In addition, the large differences in growth rates and growth requirements of the different species present in the human gut indicate that quantification by culture is bound to be inaccurate. To overcome the problems of culture, techniques based on 16S ribosomal DNA (rDNA) genes were developed.<sup>10,11</sup> These include fluorescent in situ hybridization,<sup>12-16</sup> denaturing gradient gel electrophoresis,<sup>17,18</sup> and temperature gradient gel electrophoresis. These techniques have low sensitivities and are laborious and technically demanding.

Another problematic issue 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 and 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 eliminates this problem by placing the specimen in Formalin vials for transport. This immediately kills all organisms, freezing the exact balance present at the time of collection. Since PCR identification is only looking for the genes of the microbiota, living specimens are not necessary. This allows the clinician to develop the most appropriate therapy based on the patients true gut microbiota, resulting in better clinical results.

### **Polymerase Chain Reaction (PCR)**

One of the most important and profound contributions to molecular biology is the advent of the polymerase chain reaction (PCR). PCR, one of the most significant advances in DNA and RNA-based technologies, is a powerful tool enabling us to detect a single genome of an infectious agent in any body fluid with improved accuracy and sensitivity. Many infectious agents that are missed by routine cultures, serological assays, DNA probes, and Southern blot hybridizations can be detected by PCR. Therefore, PCR-based tests are best suited for the clinical and epidemiological investigation of pathogenic bacteria and viruses. The introduction of PCR in the late 1980's dominated the clinical market because it was superior to all previously used culture techniques and the more recently developed DNA probes and kits. PCR-based tests are several orders of magnitude more sensitive than those based on direct hybridization with the DNA probe. 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. The sensitivity of the PCR test is also based on the fact that PCR methodology requires only 1-5 cells for detection, whereas a positive culture requires an inoculum equivalent to about 1000 to 5000 cells, making PCR the most sensitive detection method available.<sup>20</sup>

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

- Ability to detect non-viable 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., mycobacteria and fungi)
- Ability to detect previously unknown organisms directly in clinical specimens by using broad range primers
- Ability to quantitate infectious organisms' burden in patient specimens for better clinical responsiveness

Laboratories that make the transition to molecular diagnostics will become a more integral part of hospital operations, as they can prove the value of their improved services. The clinical microbiology laboratory is transitioning into the molecular age. From rapid pathogen and antibiotic resistance identification to screening tests, rapid molecular diagnostics are playing an increasingly important role in diagnosing and preventing infections and improving overall hospital operations. As physicians, pharmacists and even hospitals administrators demand rapid microbiology results, many laboratories are focusing on being part of cross-functional implementation teams that not only assure the new tests are implemented efficiently, but that the results af-

fect real change for patient management, hospital operations and laboratory efficacy.

### **Parasitology**

Parasitology is yet another field of microbiology to be greatly improved with molecular technologies. Parasite infections are a major cause of nonviral diarrhea even in developed countries. Classically, parasites have been identified by microscopy and enzyme immunoassays.<sup>21</sup> In recent studies, molecular techniques have proven to be more sensitive and specific than classic laboratory methods.<sup>21-23</sup> 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.<sup>22</sup> 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 combined with the need for very low amounts of genomic DNA (as low as 2.5 cells per gram),<sup>22</sup> the previously long specimen collection process, laborious and technically challenging microscopy and resulting delays in reporting have been alleviated. With PCR technology, only one fecal sample is all that is needed for 100% sensitivity and specificity in parasitology examinations.

### **Detection of antibiotic-resistance genes**

The development of bacterial resistance to antibiotic drugs involves an active change or mutation in the microbial genome which changes the microbe's metabolic or structural responsiveness to the mechanism of the drugs action. This genetic change is passed in the population as cells replicate. This genetic material can also be passed on to other strains of bacteria through plasmid sharing. The development of antibiotic resistance is becoming a serious public health issue as overuse of antibiotics continually selects for mutated strains which have developed resistance.

The human intestinal microbiota represent over 400 species. All antibiotic resistance strategies that bacteria develop are encoded in one or more genes. These genes are readily shared among and across species and genera and even among distantly related bacteria. These genes confer resistance to different classes of drugs and their sequences are known. Therefore, using PCR techniques, they can be readily detected in large populations like those found in fecal material.

The knowledge of the presence of a drug resistance gene may be quite significant for the clinician when considering treatment of a patient for a pathogen infection. For example, suppose a pathogen is detected in a stool analysis. An analysis of the presence of antibiotic resistance genes is also performed

on the sample. Subsequent drug sensitivities are then run on the pathogen and it is found to be sensitive to two antibiotics. But suppose there is also a drug resistant gene present in the sample to one of the drugs (a very possible scenario). It would be imperative, then, that this drug not be used in treating the patient. Otherwise, even though the pathogen is killed, the other organisms which have the gene conferring resistance to the drug would thrive relative to other microbes present. This would set up a potentially dangerous situation where antibiotic resistance is maintained in the population, because that gene can be readily spread to other organisms present in the individual, as well as the environment.<sup>24-26</sup> Knowledge of the presence of antibiotic resistance genes in fecal specimens, therefore, represents a significant advance in the treatment of patients and maintenance of health.

### ***Intestinal microbiota associated with obesity***

There are two predominant bacterial groups in the human GI tract, Bacteroidetes and Firmicutes. Recent research has discovered a relationship between the balance of these groups and obesity. Gastrointestinal bacteria have evolved, in a sense, independently from the host organism, in some cases performing functions which the host has consequently not had to evolve itself. One of these functions is the breakdown of dietary polysaccharides for conversion into energy.

Firmicutes bacteria, which include Bacillus, Clostridia, and Lactobacillus species, are very efficient at metabolizing plant polysaccharides into monosaccharides and short chain fatty acids. These can then 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. The Bacteroidetes group, which include Bacteroides and Prevotella species, are not as efficient in this function. Consequently, the balance of these two groups, it has been found, can significantly affect the accumulation of fat stores in the body. While obesity ultimately is caused by excess caloric intake, differences in gut microbial ecology may be an important component of energy homeostasis. In effect, obese individuals may have populations of microbiota that force a more efficient extraction and storage of energy than lean individuals possessing a different balance of microbiota.

A Bacteroidetes species decrease relative to Firmicutes in the gut has been associated with significant accumulation of body fat both in humans and experimental animals. When germ-free mice are inoculated with this imbalance of microbiota, they have significantly greater accumulations of total body fat and increased insulin resistance. Similar animals inoculated with the

better balance remain lean, even though they are eating diets that are exactly the same. Obese versus lean humans also show similar make up in bacterial groups. Lean individuals have a higher percentage of Bacteroidetes relative to Firmicutes than do obese individuals. Interestingly, if obese humans are put on low carbohydrate or low fat diets and lose weight, their microbial balance also improves.<sup>27-30</sup>

This concept of microbiota being linked to obesity raises some interesting possibilities. Studies are ongoing to explore this relationship. The use of specific diets or pre and probiotic therapies may be able to significantly affect microbial balances that affect fat storage. 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.

### ***Conclusion***

DNA analysis technology allows for a significant advancement in understanding of how GI tract microbiota affect 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 the preferred method of stool analysis.

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