

A Molecular Revolution in the Study of Intestinal Microbiota

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In recent years there has been a rapid emergence of what is now commonly referred to as 'culture-independent' techniques for the assessment of microbiota in fecal samples. Culture-independent techniques is the term given to microbiology methods that combine the use of molecular biology and genetics to identify and characterize genetic material from complex microbial environments. Such methods have replaced traditional culture techniques that use viable counting of colonies and biochemical methods to identify organisms.¹

Searches of the medical literature reveal literally thousands of scientific papers in which culture-independent techniques have been employed to measure microbiota not only in fecal samples but in multiple human, animal, food and aquatic samples. The rapid acceptance and development of culture-independent techniques has been due largely to the increased sensitivity, specificity and accuracy that such methods offer over the traditional techniques of culture and microscopy.²

Fecal Molecular Techniques Explained

Polymerase Chain Reaction (PCR) is a technology that allows specific isolation and amplification of a particular sequence of ribosomal DNA (rDNA). The rDNA gene

has regions that are identical for all bacteria, protozoa or fungi, and regions of variability that are specific for particular groups and species. Within these variable regions there are also small areas of hypervariability that may be unique for different strains of the same organism. As a result, rDNA sequences can be used to identify different species and strains of particular species within complex mixed bacterial communities.¹ The application of PCR to rDNA sequences of microbiota has found use in a range of applications from water treatment management to pre-hospitalization screening for infectious bacteria such as *Staphylococcus aureus*.³

For the next few months our *Clinical Insight* newsletter will include a regular feature on one of the exciting applications of PCR in the detection of microbiota in stool. Areas to be covered include: detection of anaerobic, opportunistic and pathogenic bacteria; detection of yeast and mold; and detection of parasites.

Common Issues With Parasite Detection

Detection of parasites in stool has traditionally presented challenges for microbiologists. Certain parasites (i.e., *Giardia lamblia*) excrete their cysts or ova irregularly,⁴ whilst other parasites (i.e. *Blastocystis hominis*) have polymorphic characteristics⁵ that make identification by microscopy a challenge. Moreover, certain parasites (i.e. *Dientamoeba fragilis*) lack a cyst stage, meaning microscopic examination has to be performed on freshly passed stool or by the use of fixatives and permanent stains to

detect the fragile trophozoites.⁶

This issue will focus on the application of PCR to detection of the well known pathogenic parasite, *Entamoeba histolytica*.

Detection of *Entamoeba histolytica*

Entamoeba histolytica is a well-documented pathogenic parasite that is the causative agent of Amebiasis. Globally, it is considered a leading parasitic cause of human mortality.⁷⁻⁹ Clinical features of Amebiasis due to *E. histolytica* range from asymptomatic colonization to amebic dysentery and invasive extraintestinal amebiasis, which is manifested most commonly in the form of liver abscesses.

E. histolytica is the only species within the genus *Entamoeba* that is associated with pathological sequelae in humans. Of the other *Entamoeba* species, *Entamoeba dispar* and *Entamoeba moshkovskii* are the most well documented. The cluster of *E. histolytica*, *E. dispar* and *E. moshkovskii* is commonly referred to as the E-Complex.

The E-Complex Dilemma

The E-Complex has presented a significant dilemma to microbiologists trying to detect pathogenic *E. histolytica*. This is due to the fact that the species which make up the E-Complex (i.e. *E. histolytica*, *E. dispar* and *E. moshkovskii*) are indistinguishable on a morphological basis. Researchers from Sydney's St Vincent Hospital and the University of Technology (UTS) have published a number of studies on the detection and differentiation of *Entamoeba* species that make up the E-Complex.¹⁰⁻¹³

Dr Stark from the Department of Microbiology at St Vincent's Hospital describes the challenges with traditional microscopy techniques for detection of *E. histolytica* in a recent paper.

*"The diagnosis of E. histolytica infection has traditionally relied upon microscopic examination of fresh or fixed stool specimens. However, microscopy has several limitations, most importantly, the inability to distinguish the pathogenic species E. histolytica from the morphologically identical nonpathogenic species E. dispar and E. moshkovskii. The sensitivity of microscopy is approximately 60% and is confounded with false positives due to misidentification of the other morphologically similar Entamoeba species. It is important to correctly diagnose patients not only to reduce the morbidity and mortality of amebiasis but also to minimize the undue treatment of patients infected with E. dispar and E. moshkovskii with antiamebic therapy."*¹²

PCR vs Microscopy

To test the ability of PCR to detect and differentiate between *Entamoeba* species, researchers from St Vincent's Hospital and UTS tested a total of 5,291 stool samples by microscopy and PCR.¹³ Of the 177 microscopy-positive E-Complex samples, only 110 were further studied as the rest of the samples were discarded because they could not be preserved. When these 110 samples were subjected to PCR, 21 (19%) were found to be negative. Of the PCR-positive samples, 3 (3.4%) were shown to contain only *E. histolytica*, 30 (33.7%) contained *E. dispar*, and 22 (24.7%)

contained only *E. moshkovskii*. Mixed infection with *E. dispar* and *E. moshkovskii* was found in 32 (36%) specimens. One sample contained both *E. histolytica* and *E. dispar*, while another sample contained both *E. histolytica* and *E. moshkovskii*. This study highlights how important PCR techniques are to the accurate detection of *E. Histolytica* and to obviate unnecessary treatment of patients infected with *E. dispar* and *E. moshkovskii*.

PCR vs Stool Antigen Detection Kits

Limitations of microscopy for the detection of *E. histolytica* have led to the development of antigen-based enzyme-linked immunosorbent assays (ELISAs). In their most recent publication, researchers from St Vincent Hospital and UTS, compared stool antigen detection kits to PCR for diagnosis of Amebiasis.¹² The findings of the study are quoted below.

"The E. histolytica PCR was found to be both sensitive and specific for the detection and differentiation of the E-Complex. In addition, the PCR was found to have a lower limit of detection of approximately one trophozoite per well. In contrast, both of the stool antigen kits (the Entamoeba CELISA PATH kit and the TechLab E. histolytica II kit) showed poor sensitivities of 28% and 0%, respectively, compared to PCR..."

Studies similar to the ones highlighted above have been replicated by other researchers around the world.^{14, 15} They provide a fascinating insight as to the effect of technological advancements on clinical disease management and diagnosis.

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A Next Generation Technology from Metamatrix! – GI Effects Stool Profiles using DNA analysis to identify microbiota

Stool Testing Comparison Chart	Glfx	Old Stool Analysis
DNA identification of microbiota	YES	NO
One sample collection per patient (even for parasites!)	YES	NO
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 slgA and total slgA	YES	?
Testing errors due to selective microbial growth during specimen transport	NO	YES
Additional costs for reflex and/or add-on testing	NO	YES



GI_{fx} GI Effects Stool Profiles

OLD STOOL TECHNOLOGY

2007: DNA technologies applied to stool testing in the GI Effects Profiles

2003: Human Genome complete

1979: Mullis invents PCR

1953: Watson and Crick discover the double-helical structure of DNA

1860's: Nucleic acids are discovered

GI_{fx}

Stool analysis expanded for digestive markers 1970's

Pasteur advances bacteriology 1860's

The Gastrointestinal Function Profile: \$399

Measures...

Beneficial/ Predominant bacteria	Inflammatory markers
Opportunistic bacteria	Immunology markers
Pathogenic bacteria	Digestion and absorption markers
Yeast/ Fungi	pH
Parasites	Occult blood, RBCs and colour
Adiposity index	Sensitivity analysis for pharmaceutical and botanical medicine interventions
Drug resistance genes	
Beneficial short chain fatty acids	

The Microbial Ecology Profile: \$230

Measures...

Beneficial bacteria	Adiposity index
Opportunistic bacteria	Drug resistance genes
Pathogenic bacteria	Sensitivity analysis for pharmaceutical and botanical medicine interventions
Yeast/ Fungi	
Parasites	

Parasatology Profile: \$150

Measures...

All known pathogenic parasites including...	Strongyloides stercoralis-nematode
Entamoeba coli	Taenia sp.
Giardia intestinalis (Iamblia)	Tapeworm
Cryptosporidium sp.	Trichuris sp.
Chilomastix mesnili	Blastocystis hominis
Dientamoeba fragilis	Giardia sp.
Endolimax nana	Necator americanus (hookworm)
Entamoeba hartmanni	Schistosoma sp.
Entamoeba dispar	Schistosoma mansoni-trematode
Iodamoeba butschlii	Strongyloides sp.
Trichomonas hominis	Taenia solium
Ascaris lumbricoides	Trichomonadinae (Trichomonas) sp.
Clonorchis sinensis	Trichuris trichiura
Entamoeba sp.	Entamoeba histolytica

Mycology: \$150

Measures...

Comprehensive list of yeast/ fungi genus and species including...	Saccharomyces sp.
Candida sp.	Taxonomy Unknown
Candida krusei	Acremonium sp.
Candida tropicalis	Aspergillus sp.
Candida albicans	Blastoschizomyces
Geotrichum sp.	Fusarium sp.
Saccharomyces cerevisiae	Paecilomyces
Trichosporon sp.	Scedosporium
Rhodotorula acheniorum	Sensitivity analysis for pharmaceutical and botanical medicine interventions

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Case Study

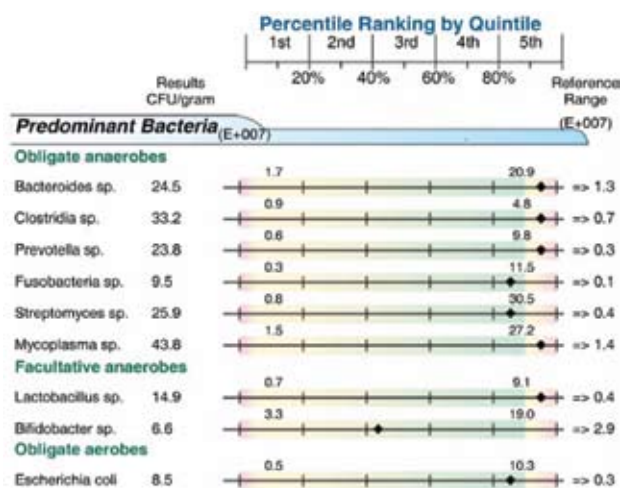
Jeremy Chatterton, B.HSc, Dip. Nutrition, Dip. Botanical Medicine

46-year-old female

A 46-year-old female presented with Chronic Fatigue Syndrome (CFS) and Irritable Bowel Syndrome (IBS). She was a practicing engineer who had been unable to work since mid 2007 due to a progressive decline in her health over a 7-year period, ever since the birth of 2 children and 4 years of frequent long-term antibiotic use for severe dental abscesses. Prior medical assessments and investigations had all been normal.

Findings

Investigations were organized and the Metamatrix GI Function Profile revealed predominant bacteria counts to be elevated. Such overgrowth is common when there is co-existing overgrowth of an opportunistic aerobic organism such as *Morganella morganii*. *M. morganii* is a commensal of the intestinal tract of humans but is an uncommon cause of infections. *M. morganii* is most often encountered in postoperative patients and is mainly associated with urinary tract infections. High levels in asymptomatic individuals usually signal dysbiosis.



Opportunistic Bacteria
Morganella morganii 2.8E+008 H <=> 1.0E+005

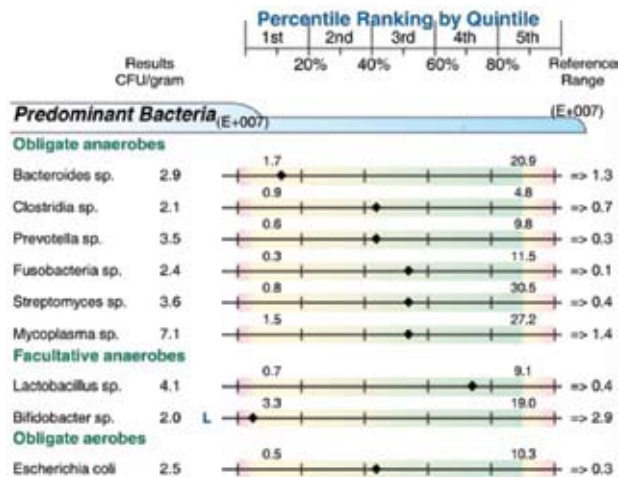
	Sensitive	Resistant
Pharmaceuticals		
Amoxicillin	S	
Ampicillin	S	
Cefuroxime	S	
Ciprofloxacin	S	
Clindamycin		R
Erythromycin	S	
Levofloxacin	S	
Penicillin		R
Potassium Clavula		R
Sulfamethoxazole		R
Tetracyclin	S	
Trimethoprim-Sulla	S	
Botanicals		
Berberine		R
Octanoic (Caprylic acid)		R

She was found to have the following parasites: *Cryptosporidium sp.*, *Blastocystis hominis*, *Giardia sp.* and *Strongyloides sp.*. An important point to note is that for each of these parasites, studies have been published that document the increased sensitivity of PCR methods

over culture and microscopy for detection.¹⁻³ The prescribing clinician has also documented higher detection rates of parasites than when using traditional stool testing. It is reasonable to suspect therefore that some or all of these parasites may not have been detected using traditional methods.

Parasitic Protozoans		
<i>Blastocystis hominis</i>	Positive	Neg
<i>Cryptosporidium sp.</i>	Positive	Neg
<i>Giardia sp.</i>	Positive	Neg
<i>Strongyloides sp.</i>	Positive	Neg

Cryptosporidium and *Giardia* are definite pathogens. The *Strongyloides* parasite has a complex life cycle in humans that involves migration of larvae from the skin to the alveoli where they are swallowed, allowing them to mature in the intestine. They are usually asymptomatic or may produce mild symptoms, but may also be life-threatening in immunocompromised hosts. *Blastocystis hominis* is thought to usually be non-pathogenic, although there are studies that demonstrate resolution of IBS following successful clearance of this organism. It is now being postulated that there may be pathogenic strains. (see below)



Parasitic Protozoans		
<i>Blastocystis hominis</i>	Positive	Neg

She was treated with anti-parasitics, using a protocol from the Centre for Digestive Disease in Sydney of 10 days of Secnidazole, Furazolidine and Nitoxoxanide combined with a herbal anti-parasitic 3 bd. The doctor felt this combination should also clear the *M. morganii*. This was followed by a course of Ivermectin (12mg stat) for the *Strongyloides*. Repeat testing showed clearance of all but the *Blastocystis hominis*. Although there has been a change in her IBS, the CFS is currently unchanged.

Persistence of *Blastocystis hominis* with no relative change to her CFS suggests the patient may be harbouring one of the more pathogenic strains of *Blastocystis hominis*. Above you can also see the suppressive effect of the anti-parasitics on predominant bacteria levels. The patient has since relocated so further follow-up has not been possible.

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