

DOCUMENTED LIMITATIONS OF CULTURE BASED STOOL ASSESSMENT

INTRODUCTION

In the last decade, DNA sequencing has played a pivotal role in our knowledge on microbial diversity providing an outstanding tool for the detection, identification, and characterization of microorganisms.^{1,2} DNA assessment has greater efficiency, reliability, and reproducibility, as well as providing both qualitative and quantitative data. DNA analysis is the new gold standard for identification of bacteria in clinical microbiology and has greatly facilitated the identification and classification of intestinal microbiota composition.³⁻⁷ Much of this has been done with techniques based on 16S rDNA gene, which include fluorescent in situ hybridization, denaturing gradient gel electrophoresis, and temperature gradient gel electrophoresis.^{4,8-10}

DNA ANALYSIS IS THE NEW GOLD STANDARD FOR IDENTIFICATION OF BACTERIA IN CLINICAL MICROBIOLOGY AND HAS GREATLY FACILITATED THE IDENTIFICATION AND CLASSIFICATION OF INTESTINAL MICROBIOTA COMPOSITION.³⁻⁷

So why does anyone use the culture method? Conventional bacteriological methods, such as microscopy and culture, have been used for analysis and/or quantification of the intestinal microbiota for decades.⁸⁻¹⁰ Barriers, such as investment in new equipment and staff, as well as commitments to on-going analysis and research collaborations, interfere with what many laboratories are able to offer. Translating science to accepted standard clinical protocol takes time.¹¹ Culture is still readily available and reasonably accurate, so clinicians continue to use it even though there are known significant limitations which include: transport issues, indeterminate and presumptive quantification and identification, as well as subjectively demanding techniques.^{12,13} In this review, we discuss six major pitfalls of conventional stool assessment.

I. TRANSPORT ISSUES

The primary issue with culture analysis is that of transport. Since analysis is culture dependent, sample collection must be done using nutrient broth containers to keep as many bacteria alive as possible in transport. This broth allows continued growth of those species most satisfied with the broth medium during transport, until the sample is finally plated for culture. This growth results in 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. Formalin immediately kills all organisms, freezing the exact balance present at the time of collection. Since DNA identification is only looking for the genes of the microbiota, living specimens are not necessary. This technique allows the clinician to develop the most appropriate therapy based on the patient's true gut microbiota, resulting in better clinical results.

Recent studies in our laboratory illustrate this situation. One specimen was placed in two vials: one vial containing formalin and another a nutrient broth commonly used for transport. Both vials were incubated for three days at room temperature, then DNA was extracted. The extract was incubated with three different restriction enzymes which cleave the DNA at specific base pair sequences (Figure 1).

Figure 1

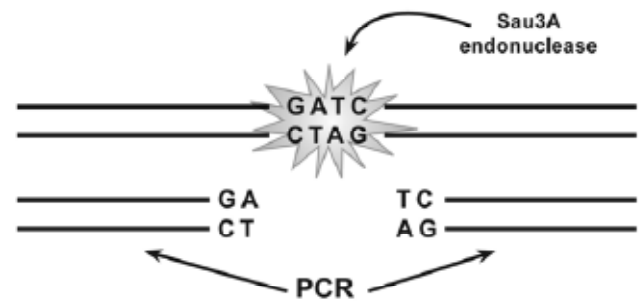


Figure 1. A restriction enzyme or restriction endonuclease is an enzyme that cuts double-stranded DNA when recognizing a specific short nucleotide sequence. The restriction enzyme Sau3A endonuclease cleaves DNA strands when it finds the sequence 5' GATC and 3' CTAG. These fragments can then be amplified using PCR.

The digestate was amplified by polymerase chain reaction (PCR), and then placed on an agarose gel plate and the DNA fragments were electrophoretically separated. Since restriction enzymes cleave the DNA only at specific base pair sequences, broths with identical populations of microbes would produce the same patterns of banding in the electrophoretic runs. If, however, there were different amounts and types of microbiota in the two tubes, then differences would appear in the banding patterns. This was clearly demonstrated in the experiment (Figure 2).

Figure 2

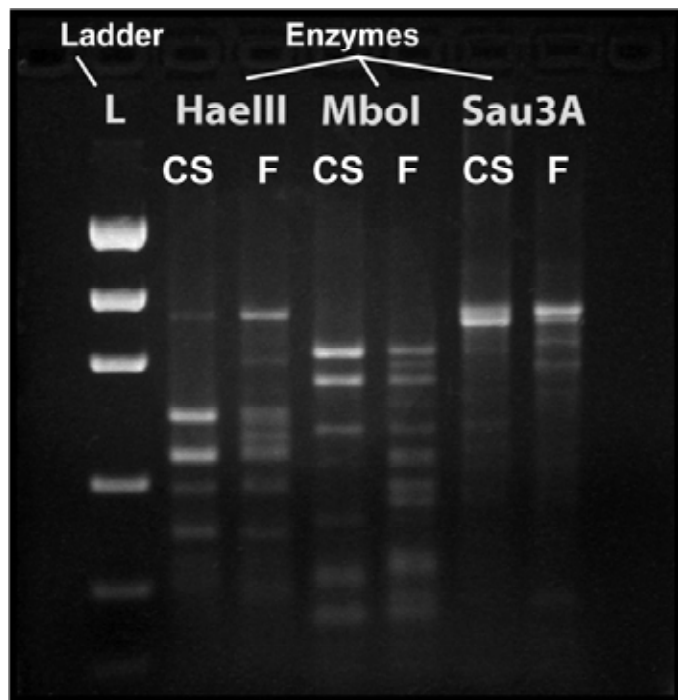


Figure 2. Three restriction enzymes HAEIII, MboI, and Sau3A digest the same two samples. One sample comes from culture and sensitivity (CS) vial containing a nutrient broth. The other sample vial contains formalin (F). Both vials have incubated for three days. The banding patterns are significantly different for the two vials indicating the populations of microbiota present are significantly different. In addition, note the CS vials have much fewer and heavier bands indicating that the microbial populations have reduced diversity due to the anaerobic die off and those viable ones have overgrown. Some bands present in the F vials are not detected in the CS vials where whole groups have died off. L is a calibration of known DNA sizes.

Another observation from the data is the loss of banding in the nutrient broth vial over time, indicating overgrowth of aerobes at the expense of the anaerobic populations and consequent loss of diversity. Opportunistic, potentially pathogenic organisms can also overgrow under these conditions. This overgrowth has been seen in our laboratory. For these reasons, nutrient broth transport vials cannot provide the specimen integrity required for accurate measurement of gut microbes.

Figures 3 through 5 illustrate the growth response of *Bifidobacteria*, *Candida* species and *Staphylococcus aureus* in one subject over three days. This data indicates that clinical laboratory data relying on transport of the specimen

to the laboratory for culture are likely erroneous and can lead to inappropriate patient treatment. *Candida* species overgrowth is particularly notable (Figure 5). As these data indicate, *Candida* proliferates in nutrient broth transport media. This overgrowth in transport has likely fueled the common belief of many practitioners of the deleterious health effects of *Candida albicans* overgrowth in the gut. This belief may have resulted in inappropriate prescription of anti-fungal agents.

Figure 3

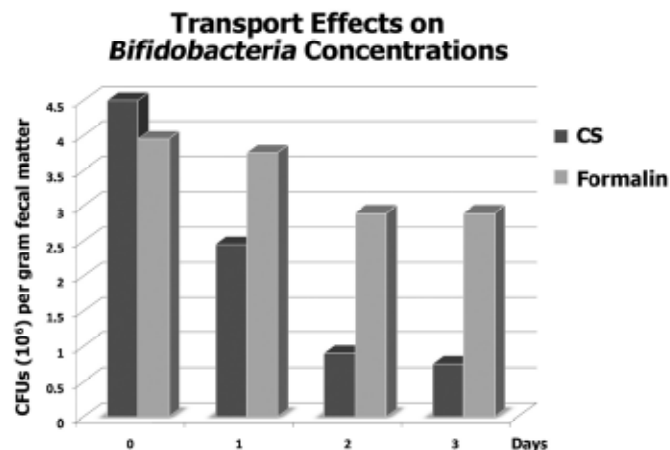


Figure 3. Significant decreases in *Bifidobacteria* occur over three days in standard nutrient broth vials (CS) used in traditional culture methodologies. Formalin-fixed samples show more consistent levels.

Figure 4

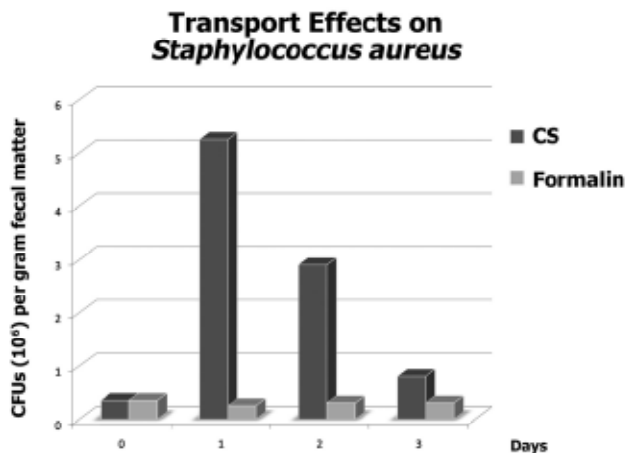


Figure 4. Potentially pathogenic, the opportunistic bacteria *Staphylococcus aureus* shows significant overgrowth during a three-day incubation in nutrient broth vial (CS). Formalin-fixed sample vial shows no growth. CFUs = colony forming units.

Figure 5

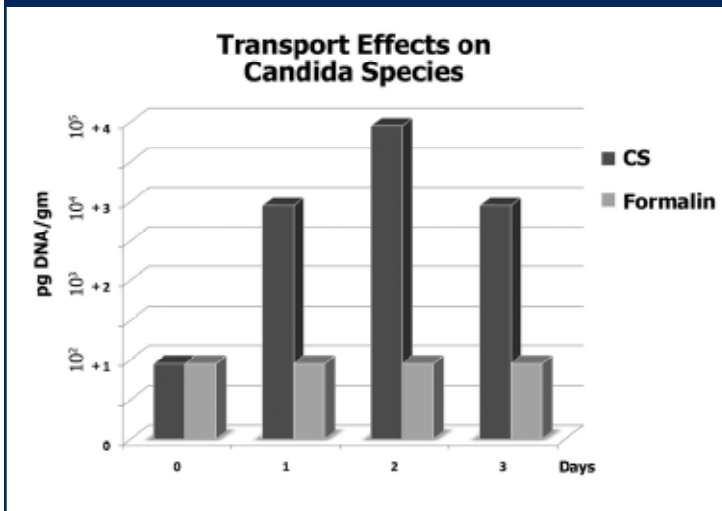


Figure 5. *Candida* species show significant overgrowth in nutrient broth vials (CS) over three days. Formalin-fixed sample vials do not exhibit growth and are at a level that would not be reported as abnormal overgrowth. The CS samples at 1, 2, and 3 days would be reported as abnormal overgrowth.

THIS DATA INDICATES THAT CLINICAL LABORATORY DATA RELYING ON TRANSPORT OF THE SPECIMEN TO THE LABORATORY FOR CULTURE ARE LIKELY ERRONEOUS AND CAN LEAD TO INAPPROPRIATE PATIENT TREATMENT.

2. QUANTITATIVE ASSESSMENT

In the Metamatrix DNA assessment, quantification of predominate, opportunistic, and pathogenic bacteria are given as exact amounts. Yeast/fungal values are given as a plus 1, 2, 3, or 4 and represent a specific value range. For example, a +1 is specifically quantified to mean 100 – 999 pica grams of DNA per gram with asystematic reproducible result. Similarly, bacterial assessments are quantitatively reported as Colony Forming Units per gram sample.

In laboratories that utilize culture assessments, values of quantity are significantly less specific or reproducible. For example, when samples come into the laboratory, they are plated onto a dish with selective or differential media. The technician dips a tool, generally a cotton tipped applicator, into the sample and puts it on a plate that is divided into four quadrants. The technician starts streaking in quadrant 1, then drags the tool to quadrant 2, 3, then 4 in sequence. The dishes are incubated overnight at which time they are

assessed. If the bacteria grew in the first quadrant only, it is called a +1. The assumption is that there was only enough of the bacteria in the sample, due to low concentrations, to grow only in the first quadrant. The first quadrant is expected to have the greatest numbers of bacteria since that is where the streaking starts. If colonies appear in all four quadrants, it is called a +4. The fourth quadrant would be expected to have the least amount of original bacteria present since it is the last streaked. Thus, if something grew in the fourth quadrant, it is assumed that it grew there because bacteria were present in high concentrations in the sample. No procedure or assessment is done to confirm this assumption. No standardization for original amount of sample on the tool exists. A +4 theoretically means that there was enough of the bacteria on the sample that it stayed on the tool while it was dragged from the first to the fourth quadrant. However, with no standardization, these steps are at best a subjective judgment by the technician.

Additionally, if growth is found in the first and fourth quadrant, but not in the second and third, some technicians may call this a +4, while another may call it a +2. These results are technician based, and allow for human error, such as the technician turning the tool differently in the last quadrant. Consequently, a +1, +2, +3, or +4 from culture is an unverified guess based on where the microbe grew in the culture dish. This is at best a qualitative assessment of bacterial presence in the sample. These discrepancies are some of the reasons hospital-based laboratories give results as positive or negative and do not attempt to quantify when using culture techniques.

3. PRESUMPTIVE DIAGNOSIS/IDENTIFICATION

To identify individual microbes growing on the initial plates, individual colonies are selected and grown out again. Using a process of elimination composed of several if-then steps, a series of tests are done which yield a diagnosis of the most likely organism present. Typical characteristics such as colony morphology, key biochemical reactions, and drug susceptibility patterns assist in establishing the suppositious identification of the microorganism. However, most labs imply that this diagnostic identification of the microbe is definitive. It is not definitive, but at best gives a percentage ‘best estimate’ of the likelihood of identification accuracy.

It is widely accepted that plate culturing techniques reveal only a small portion of the true microbial population, primarily due to an inability of detecting organisms that might not be cultivable with known existing media, temperature specifics, phases of metabolic activity, and the inability of recovering known microorganisms which are viable but enter a non-cultivable state.¹³⁻¹⁵ DNA analysis became the standard of research more than a decade ago, partially due to the subjective nature of culture ID systems. The sensitivity of conventional culture method was compared to DNA analysis for identifying *Shigella spp.* and enteroinvasive *Escherichia coli* (EIEC) in known cases of dysentery. DNA had an identification accuracy rate of 96%, compared to culture's identification rate of 54%.¹²

HOWEVER, MOST LABS IMPLY THAT THIS DIAGNOSTIC IDENTIFICATION OF THE MICROBE IS DEFINITIVE. IT IS NOT DEFINITIVE, BUT AT BEST GIVES A PERCENTAGE 'BEST ESTIMATE' OF THE LIKELIHOOD OF IDENTIFICATION ACCURACY.

4. IDENTIFICATION OF PREDOMINATE BACTERIA

Bifidobacteria are among the first to establish themselves in the infant's intestinal tract, and in the healthy infant, they predominate. While it would be impossible under normal life circumstances to have 0+ as a level of *Bifidobacteria*, that level is often reported in laboratories utilizing culture techniques. In these laboratories, *Lactobacillus* and *Bifidobacteria*, both facultative anaerobes, are identified primarily based on selective growth media. Though further enzyme tests such as coagulase or catalase may be done to hone the identification of *Lactobacillus*, *Bifidobacteria* identification is done solely on this single growth. Quantification is also made from the growth on these plates, as described above. These phenotypic methods suffer from a lack of reproducibility generated by conditions of culture used in different laboratories and to the diversity of strains that comprise the recognized species.¹⁶ DNA identification and quantification of *Lactobacillus* and *Bifidobacteria* is exact.

5. PARASITOLOGY

Parasitology is yet another field of microbiology to be greatly improved by molecular technologies. Classically, parasites have been identified by microscopy and enzyme immunoassays.¹⁷ In recent studies, DNA techniques have

proven to be more sensitive and specific than classic laboratory methods.^{4, 17, 18} One example is *Giardia*. Since *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.⁴ Even with the advent of antigen detection systems, there has long been uncertainty in diagnosis when no ova or parasites are found. With PCR technology, only one fecal sample is needed for 100% sensitivity and specificity in parasitology examinations. The clinician needs to be aware that assessment of parasites, while good by microscopy, is much more accurate with DNA based technologies.

6. AUTOMATED IDENTIFICATION SYSTEMS

There are three main automated systems in use for microbial identification: the MicroScan, VITEK 2, and Crystal GP. All use the if-then sequences discussed earlier to identify characteristics of microbiota and produce proposed bacterial identification with a given accuracy expressed as a percentage.

The accuracy of these systems was evaluated by identifying coagulase-negative staphylococci (CNS). Verification was done with DNA by 16S rRNA sequencing. The MicroScan, VITEK 2, and Crystal GP systems correctly identified 82.5%, 87.5%, and 67.5% of the isolates, respectively. Misidentification was the main problem in MicroScan and Crystal GP systems, whereas the main problem of VITEK 2 was low-level discrimination. None of the three phenotypic systems tested could accurately and reliably identify CNS at the species level as well as the 16S rRNA technique.¹⁹ There are two types of the VITEK systems, the VITEK 2 fluorescent card and the VITEK 2 colorimetric card assay.²⁰ In another study, the accuracy of identification at the species level for the VITEK 2 fluorescent card, the VITEK 2 colorimetric card assays, and the API 20 NE (another identification system), were 48%, 59%, and 46%, respectively.²¹ In comparing 16S rRNA gene sequencing to the VITEK 2 fluorescent card, the VITEK 2 only identified 43% of the isolates correctly at the species level, a low rate.^{21, 22} Due to this low rate of identification, the authors proposed an algorithm for proper identification of non-fermenting gram-negative rods. In the algorithm, isolates with only good or acceptable identification to species level should be subjected to 16S rRNA gene sequencing when accurate species assignment is sought.²²

CONCLUSION

DNA assessment is specific and accurate in the identification and quantification of fecal microbiota. As discussed above, culture methods are relatively inefficient, time consuming, and labor intensive, often leaving diagnosis obscure. DNA is highly sensitive and specific. Beginning with the sample collection, DNA assessment is superior because it avoids the pitfalls of transport that culture methods suffer from and allows the identification of true gut microbiota, as well as the anaerobic population comprising 95% of gut microbiota. The results are the actual types and amounts at the time of collection, not what survived in a growth medium. DNA further reports the microbiota as true numbers, values that were specifically quantified, not an assumption. The ability of DNA to accurately identify microbiota has been one of the primary reasons why it has been so embraced by the research community for the last decade. Research on *Bifidobacteria* and *Lactobacillus* has significantly expanded since the introduction of DNA analysis. DNA techniques are known to be more sensitive and specific than classic laboratory methods.^{4, 17, 18} Verification of the accuracy of culture assessment tools and equipment is done by comparing their results to the “actual” value, which is determined by DNA assessments.

THOUGH CULTURE HAS BEEN CORRELATED TO CLINICAL FEATURES IN OLDER RESEARCH STUDIES, WE NOW KNOW MUCH OF THE DATA WAS INCORRECT.

Sellers of conventional stool tests argue that DNA assessment does not have the years of clinical background that culture has. This point of course is misleading. Though culture has been correlated to clinical features in older research studies, we now know much of the data was incorrect, due to the significant limitations of culture and the indeterminate results it produces. Researchers have been actively reevaluating past research for this reason. Specific values of gut bacteria have been identified for normal healthy populations using DNA assessments, as well as for populations of selected groups and specific disease conditions.^{7, 23}

A notable example many clinicians are now aware of is the research of Dr. Gordon and Dr. Ley who discovered that

obese volunteers had greater amounts of Firmicutes and significantly less Bacteroidetes than the lean participants.^{24, 25} In their research on fat bugs, they performed a comparative 16S-rRNA-gene sequence-based survey (DNA analysis) of the distal gut microbiota - NOT CULTURE. Gordon's team could not and would not have done this research with culture, because it cannot produce this information. DNA analysis is the most thorough and reliable assessment of the gut microbiota. It eliminates the major limitations of conventional stool testing using culture methods and offers advanced knowledge for more effective patient treatment.

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