Assessment of the Diagnostic Accuracy of Recently Introduced DNA Stool Screening Test

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INTRODUCTION

The "gold standard" for detection of enteric pathogens in stool samples is bacterial culture using a variety of selective and differential media. However, culture methods can require several days to complete and are targeted for the detection of bacteria that can be grown in culture. There is need for qualitative and quantitative tests that are more rapid than bacterial culture. Real-time detection polymerase chain reaction (RTD-PCR) has been applied for the detection of food-borne pathogens (12), cancer (3,7,11), genetic diseases (20) and infectious diseases (6,8,10,13). This method produced a linear quantitative detection range of 7 logs, with a lower detection limit of 10^3 colony-forming units (CFU)/g tissue or a few copies per reaction. (14)

In 2007, a diagnostic testing laboratory ("Subject Laboratory") began offering a stool-screening test that uses a proprietary DNA method to identify gut microbiota including anaerobes. The Subject Laboratory claims that their DNA assessment is specific, accurate, avoids the pitfalls of sample transport, reports results as specific numbers, and is

more sensitive than classic laboratory methods. Their stated cutoff for clinically significant pathogens is 1 x 10^3 organisms/gram. The purpose of this study was to assess the accuracy and specificity of this new testing modality by conducting a proficiency analysis study performed by an independent Life Sciences research organization (IIT Research Institute [IITRI], Chicago, IL).

MATERIALS AND METHODS

Stool Inoculation, Human stool was utilized as a matrix in which to spike known concentrations of various bacterial pathogens. All samples were prepared from a human stool pool that served as the consistent control matrix for all samples. This matrix also provided a background of normal stool flora and was used throughout the study. The test platforms were the Subject Laboratory's Specimen Collection Kits that were prepared as instructed by the package inserts. One gram of stool was added to each of three vials containing either C&S Medium, 10% Formalin Fixative, or Nucleic Acid Collection Solution. Each vial was subsequently spiked with 0.1mL of bacterial target concentrations at either approximately 1.0 x 10^7CFU/mL or 1.0 x 10^4 CFU/mL. All samples including the normal unaltered stool specimen were shipped to the Subject Laboratory via overnight courier the same day they were prepared with a request for stool analysis.

Bacteria Used. Cryovials containing frozen aliquots 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 utilized. Bacterial preparations were made after aseptically inoculating bacteria into 25 mL of Trypticase Soy Broth. *S. sonnei, S. typhi, E. coli, V. parahemolyticus, A. hydrophilia, P. shigelloides, E. tarda,* and *Y. enterolytica* spiked broths were incubated overnight at 37 ± 2°C overnight. *C. jejuni* and *C. difficile* broths were cultured in anaerobic jars with BD GasPaks™ for 2-3 days at 40 ± 2°C and for 2 days at 37 ± 2°C, respectively.

Colony Counts. Each overnight incubated culture was diluted in 0.1% peptone to a concentration of approximately 1.0 x 10^7 colony forming units/mL (CFU/mL) using McFarland standardization. Serial dilutions were plated in quintuplicate to confirm the concentration of the spike-aliquots. Titer plates were incubated for the various bacteria as described.

RESULTS

A total of 34 stool samples were sent for Stool Testing. 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. Thirty-one specimens were spiked with bacterial pathogens at clinically significant levels that are within the sensitivity of culture based methods, and at higher levels well above the Subject Laboratory's reported lower limit for detection of pathogens. Three "control" specimens were unaltered and contained no bacterial, fungal or parasitic pathogens. All 31 stool specimens containing bacterial pathogens were reported negative for the indicated pathogens by the Subject Laboratory. Seventeen samples were reported as "Parasite present, taxonomy unavailable." Fifteen samples from the same stool specimen were reported as "No Ova or Parasites." One specimen was reported to contain *Cryptosporidium* sp. and one specimen was reported to contain *Enterobius vermicularis*. Two of the samples that were reported to contain "Parasite present, taxonomy unavailable," were also reported to contain *Cryptosporidium* sp. Complete results are shown in Table 1.

Table 1. Results of Stool analysis Conducted by Subject Laboratory: (-) bacteria not present; (+) bacteria present

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

Table 1. Results of Stool analysis Conducted by Subject Laboratory—continued							
Sample ID	**Organism Added to Normal Stool Specimen**	**Quantity**	**Normal Stool Flora**	**Opportunistic Bacteria**	**Pathogenic Bacteria**	**Yeast/ Fungi**	**Parasites**
3	Salmonella typhi	4.4x102 CFU/g	+	-	-	-	Parasite Present; taxonomy unavailable
4	Salmonella typhi	4.4x105 CFU/g	+	-	-	4+ => 1000000pg DNA/g specimen Geotricum sp.	No Ova or Parasites
5	E. coli 0157:H7	2.8x102 CFU/g	+	-	-	-	Cryptosporidium sp. Positive, Parasite Present; taxonomy unavailable
6	E. coli 0157:H7	2.8x105 CFU/g	+	-	-	-	Parasite Present; taxonomy unavailable
7	Campylobacter jejuni	2.8x102 CFU/g	+	-	-	-	Parasite Present; taxonomy unavailable
8	Campylobacter jejuni	2.8x105 CFU/g	+	7.3 X 107 Bacillus sp.	-	-	No Ova or Parasites
9	Vibrio parahemolyticus	5.8x101 CFU/g	+	-	-	-	Parasite Present; taxonomy unavailable
10	Vibrio parahemolyticus	5.8x104 CFU/g	+	-	-	-	Cryptosporidium sp. Positive
11	Aeromonas caviae	3.4x102 CFU/g	+	-	-	-	Parasite Present; taxonomy unavailable
12	Aeromonas caviae	3.4x105 CFU/g	+	-	-	-	Parasite Present; taxonomy unavailable
13	Plesiomonas shigelloides	4.4x102 CFU/g	+	-	-	-	Parasite Present; taxonomy unavailable
14	Plesiomonas shigelloides	4.4x105 CFU/g	+	-	-	-	Enterobius vermicularis Positive
15	Edwardsiella tarda	9.5x102 CFU/g	+	-	-	-	Cryptosporidium sp. Positive, Parasite Present; taxonomy unavailable

Table 1. Results of Stool analysis Conducted by Subject Laboratory—continued							
Sample ID	**Organism Added to Normal Stool Specimen**	**Quantity**	**Normal Stool Flora**	**Opportunistic Bacteria**	**Pathogenic Bacteria**	**Yeast/ Fungi**	**Parasites**
16	Edwardsiella tarda	2.4x103 CFU/g	+	-	-	2+ => 1000pg DNA/g specimen Candida sp.	Parasite Present; taxonomy unavailable
17	Edwardsiella tarda	9.5x105 CFU/g	+	-	-	-	Parasite Present; taxonomy unavailable
18	Yersinia enterocolitica	5.0x102 CFU/g	+	1.0 X 108 Staphylococcus aureus	-	-	No Ova or Parasites
19	Yersinia enterocolitica	5.0x105 CFU/g	+	-	-	-	Parasite Present; taxonomy unavailable
20	Clostridium difficile	2.4x101 CFU/g	+	-	-	-	No Ova or Parasites
21	Clostridium difficile	2.4x104 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	6.5x103 CFU/g	+	-	-	-	No Ova or Parasites
25	Shigella sonnei	6.5x106 CFU/g	+	-	-	-	No Ova or Parasites
26	Yersinia enterocolitica	9.0x103 CFU/g	+	-	-	-	No Ova or Parasites
27	Yersinia enterocolitica	9.0x106 CFU/g	+	-	-	-	No Ova or Parasites
28	E. coli 0157:H7	5.6x103 CFU/g	+	-	-	-	Parasite Present; taxonomy unavailable
29	E. coli 0157:H7	5.6x106 CFU/g	+	-	-	-	No Ova or Parasites
30	Vibrio parahemolyticus	9.2x102 CFU/g	+	-	-	-	Parasite Present; taxonomy unavailable
31	Vibrio parahemolyticus	9.2x105 CFU/g	+	6.1 X 107 Klebsiella pneumoniae	-	-	Parasite Present; taxonomy unavailable
32	Clostridium difficile	5.4x102 CFU/g	+	-	-	-	No Ova or Parasites
33	Clostridium difficile	5.4x105 CFU/g	+	-	-	-	No Ova or Parasites
34	Normal Stool Flora	N/A	+	-	-	-	No Ova or Parasites

Table 1. Results of Stool analysis Conducted by Subject Laboratory—continued							
Sample ID	**Organism Added to Normal Stool Specimen**	**Quantity**	**Normal Stool Flora**	**Opportunistic Bacteria**	**Pathogenic Bacteria**	**Yeast/ Fungi**	**Parasites**
28	E. coli 0157:H7	5.6x103 CFU/g	+	-	-	-	Parasite Present; taxonomy unavailable
29	E. coli 0157:H7	5.6x106 CFU/g	+	-	-	-	No Ova or Parasites
30	Vibrio parahemolyticus	9.2x102 CFU/g	+	-	-	-	Parasite Present; taxonomy unavailable
31	Vibrio parahemolyticus	9.2x105 CFU/g	+	6.1 X 107 Klebsiella pneumoniae	-	-	Parasite Present; taxonomy unavailable
32	Clostridium difficile	5.4x102 CFU/g	+	-	-	-	No Ova or Parasites

DISCUSSION

There is a growing demand for faster results for microbiology testing and a growing demand for molecular based analyses that promise results on demand. However, molecular based testing for stool pathogens is still under development and there are currently no FDA cleared *in vitro* assay commercially available. In this study we challenged the claims of a CLIA licensed laboratory that offers a novel DNA method for identifying microorganisms in human stool samples. Our survey showed that the subject laboratory was unable to identify any of the ten enteric pathogens added to a normal stool specimen even though the quantities of microorganisms added were at levels above the stated threshold of detection for the novel assay. Furthermore, the subject laboratory reported "parasites present" in 50% of the samples tested even though no parasites were added to the survey samples and an equal number of the same stool sample were reported negative for parasites.

Other investigators have reported the successful application of molecular methods for detection of microorganisms from human gastrointestinal samples. Real-time PCR has been successfully applied for quantification of bacterial DNA in feces (2,9,15,19), colonic tissue (4), rumen (18), gastric tissue (5) and periodontal samples (1). 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 real-time PCR using SYBR Green I chemistry has an advantage of being a very sensitive and precise technique for an extensive quantitative evaluation of the gut microbiota and is also feasible for detection of human pathogens from fecal samples. Using fecal samples spiked with various amounts of target bacteria they demonstrated detection limits could be obtained that were between 6×10^3 (*H. pylori*) and 6×10^4 (*Clostridium difficile* and *Campylobacter jejuni*) cells per gram of feces (16). In a subsequent publication, Rinttilä et al. used quantitative real-time PCR (qPCR) panel to detect 12 pathogenic microorganisms from fecal samples of irritable bowel syndrome subjects (17).

Some laboratories have developed in-house assays and offer them commercially with the nomenclature of Lab Developed Tests (LDTs). They offer these assays under the banner of a CLIA licensed laboratory and provide a disclaimer on the patient report stating that the "Assay is not FDA cleared and results should not be used for patient diagnosis." Such is the case for the laboratory that is the subject of this study. The results from the stool analysis are labeled with the following disclaimer: "These test results are not for the diagnosis of disease. They are intended to provide nutritional guidelines to qualified healthcare professionals with full knowledge of patient history and concerns to assist in their design of an appropriate healthcare program." However, when a sample of physicians who use the Subject laboratory for stool analysis were asked if they use the results from the

Subject laboratory for patient diagnosis they all said yes and pointed to the fact the laboratory was CLIA licensed so they concluded that the test results must be valid. We should point out that there is no proficiency testing survey available for the assay that is performed by the Subject laboratory, the method being used is proprietary and has not been published and the laboratory is not willing to provide their verification study data to their clients.

Although there is a need to develop rapid molecular testing assays for characterization of the gut microbiome, physicians and patients need to be aware that all stool analysis assays may not be valid and users of these assays should demand to see verification study data in order to discern the claims of the commercial entity offering the lab developed assay. The claims made by the Subject Laboratory that their DNA assessment of stool samples is specific and accurate, could not be supported by this independently conducted proficiency challenge.

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