Biomarkers for Infectious Disease Diagnostics in the Developing World

Diagnosis of *Giardia lamblia*, *Cryptosporidium parvum*, and Enteroaggregative *Escherichia coli* in Children with Diarrhea as a Surrogate of Stunting

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In this review of diagnostic biomarkers for diarrheal diseases, Section 1 provides an introduction to the diagnostic needs for the detection of *Giardia lamblia*, *Cryptosporidium parvum*, and enteroaggregative *Escherichia coli* in children with diarrhea as a surrogate of stunting. Section 2 discusses the current status of biomarkers and technologies that have been used for the diagnosis of these pathogens in patients with diarrhea. Sections 4 through 7 outline the steps that need to be taken to improve existing biomarkers, or to identify and develop new biomarkers. Finally, in Section 8, the recommended courses of action are presented.

1. Introduction to Diagnostic Needs for Diarrheal Diseases in Resource-Limited Settings

Accurate information on the impact of diarrheal disease in most areas of the developing world is often either not available, incomplete, or outdated. However, estimates of the impact of diarrheal diseases around the world range from one to four billion diarrhea episodes every year among children younger than five years of age in socio-economically developing countries, causing 2 to 2.5 million deaths, of which about 85% occur in the poorest parts of the world.[1,2] Estimates from different areas of the world suggest that the children may have between two and eight episodes of diarrhea in the first year of life.[1] In some countries, diarrheal diseases account for over 20% of all deaths in children younger than five. This figure is particularly disturbing given that in the more economically developed world, diarrhea is associated with less than 1% of deaths in young children. Children living in resource-poorer areas have more diarrhea episodes, more severe episodes with dehydration, and a higher death rate compared to children living in resource-richer areas. The factors contributing to this are often factors related to poverty, such as deficiencies in the water and sewage infrastructure, crowding and exposure to farm animals, lower standards in food handling care and hygiene, limited access to medical care, and low levels of education. In addition, malnutrition contributes to the increased risk of death from diarrhea.[1]

Diarrhea is a manifestation of intestinal dysfunction that results in increased passage of loose or liquid stools, resulting in a loss of fluids, electrolytes, and nutrients. It is primarily a symptom of gastrointestinal infection, though non-infective causes occur as well.[2] Fever, vomiting, abdominal cramps, and dehydration of different severities can accompany diarrhea. Assessment of the characteristics of the stool can help determine whether a secretory or small intestinal dysfunction is present (usually indicated by liquid non-bloody stools), or an inflammatory or invasive process is occurring in the colon (indicated by dysenteric or bloody stools). However, neither the stool characteristics nor the clinical symptoms are sufficiently specific to allow the determination of a particular etiology. Most cases of diarrhea resolve within seven days. Persistent diarrhea lasting longer than 14 days has been associated with a variety of pathogens.

More than 20 viral, bacterial, and parasitic enteropathogens are currently associated with acute diarrhea.[1] Three of these, *Giardia lamblia*, *Cryptosporidium parvum*, and enteroaggregative *Escherichia coli* cause acute diarrhea, but are also associated with chronic diarrhea or asymptomatic infections which reduces the nutritional status of the child, and therefore can result in impaired growth and development. Repeated

and persistent diarrhea can worsen malnutrition, which in turn may make the patient more susceptible to infectious diarrhea, creating a vicious cycle in young children in developing countries.[2]

The majority of *E. coli* are usually harmless commensals of the mammalian gastrointestinal tract, but some strains have acquired specific virulence traits that enable them to cause disease in healthy individuals.[3] The clinical syndromes resulting from these altered forms of *E. coli* include gastroenteritis, urinary tract infection, septicemia, and meningitis. *E. coli* enteritis is caused by at least six distinct *E. coli* pathotypes, including enterotoxigenic (ETEC), enteropathogenic (EPEC), enterohemorrhagic (EHEC), enteroinvasive (EIEC), diffusely adherent (DAEC), and enteroaggregative *E. coli* (EAEC). These pathotypes have distinct clinical, epidemiological, and pathogenic characteristics. Strains within each pathotype often have similar virulence traits (i.e. genes) and their typing can be further refined by their O (lipopolysaccharide) and H (flagellar) antigens.[4] EAEC strains are increasingly recognized as an important cause of diarrhea world-wide, in all age groups and in developing as well as industrialized countries. EAEC may be best known for its role in persistent diarrhea in children in developing countries, and the malnutrition and impaired growth that result. Infection causes a watery diarrhea which can be inflammatory, and can be associated with abdominal pain, borborygmi, and low-grade fever.[5] EAEC transmission is thought to occur via the oral-fecal route, but the difficulties in identifying this class of pathogen have resulted in very little data, and conflicting data, on risk factors for infection or identification of environmental presence. Treatment of EAEC is complicated by the fact that isolates from diverse locations are often resistant to multiple antibiotics. Currently it appears that most strains are susceptible to ciprofloxacin.[5]

G. lamblia is a binuclear flagellated protozoan parasite with trophozoite and cyst stages. The trophozoites replicate in the small intestine, where they cause symptoms of diarrhea and malabsorption. Some of the trophozoites form cysts, which are passed in to the feces. The cysts are fairly stable under a variety of environmental conditions, and can remain infective for months under cool damp conditions.[6,7] Trophozoites are also shed in the feces, but they are not infective.[7] *G. lamblia* is spread by the fecal-oral route through ingestion of the cyst stage parasites, and both food-borne and water-borne modes of transmission have been reported.[1] Symptoms of *G. lamblia* infection (giardiasis) are dependent somewhat on the age of the patient, with diarrhea, vomiting, anorexia, and failure to thrive typically occurring in young children. In addition, infection can be asymptomatic or only mildly symptomatic. *G. lamblia* infection is typically treated with metronidazole or tinidazole.

There are a number of species in the genus *Cryptosporidium*, but the majority of human cases of illness are caused by *Cryptosporidium parvum. C. parvum* is a spore-forming coccidian protozoon. Its lifecycle results in the shedding of mature oocytes in the feces, and the "thick-walled" variety of oocytes can survive for extended periods in the environment. It is thought that during acute infection, humans shed large numbers of oocytes, but that during asymptomatic infection, smaller numbers of oocytes are shed. Oocytes are found ubiquitously in the environment, often in surface waters and shallow springs.[7] Infection spread by the fecal-oral route, either by person-to-person contact, or by water-borne or food-borne modes of transmission.[1] Symptoms of infection (cryptosporiasis) in immuno-competant individuals include profuse watery diarrhea, abdominal pain, myalgia, fever, and weight loss. In healthy individuals, the infection is self-limiting, and the symptoms usually resolve within a few weeks. In immuno-

compromised individuals and young children, the infection is more serious and can become chronic and even fatal. Typically, infection in infants results in chronic diarrhea and malnutrition. *C. parvum* infection is typically treated with nitazoxanide, and though this drug suppresses the parasite, there is no reliably effective drug treatment to clear the infection.[7]

A variety of biomarkers and diagnostic approaches have been used to diagnose the three diarrhea-causing pathogens of interest in this document, and their individual performance is reviewed in the following section. Future opportunities to detect biomarkers from all three pathogens in a single diagnostic assay are discussed in Sections 4, 6, and 8.

2. Diagnosis of EAEC, *C. parvum*, and *G. lamblia* in Patients with Diarrhea in Resource-Limited Settings: Status of Currently Available Biomarkers

In general, attempts are not made to identify the etiology of diarrhea in resource-limited settings, and therefore there are no methods that are currently used in such settings.

In moderate to high-resource settings, a wide range of biomarkers and diagnostic approaches are currently used, because there is no single diagnostic test that can diagnose bacterial, viral, and parasite pathogens that are causes of diarrhea. Traditional methods therefore utilize a panel of different assays, often microscopy and culture-based methods, to identify as many pathogens as possible, given the resources available in a particular laboratory. Though a panel of different tests is currently used, it would clearly be advantageous to implement one test, which uses one sample per patient, to diagnose all the pathogens of interest. The goal of developing a single multiplex assay is addressed again in later sections.

The ability to obtain an etiological diagnosis in a child with diarrhea using traditional methods is highly dependent on the quality of the stool sample, the experience and skill of the microbiologist, and the resources available to perform various assays that are specific for particular pathogens. There are no universal consensus guidelines for the evaluation of stool samples using culture- and microscopy-based methods, and therefore each laboratory develops their own guidelines. In most laboratories, analyses that involve a stool culture require 48 to 72 hours to perform, after the collection of the sample. In recent years, molecular diagnostic methods have been added to the traditional culture and microscopy-based methods.

The biomarkers and diagnostic approaches that are currently used to diagnose EAEC, *C. parvum* and *G. lamblia* infection are presented in the following sections.

2A. Detection of Adherent Bacteria Using Culture-Based Approaches

Adherence tests developed almost 20 years ago have been used to demonstrate that there are two types of adherent *E. coli* strains associated with diarrhea, the "diffuse" (i.e. diffusely aggregative) and the "aggregative" strains. Aggregative adherence is characterized by what is often termed a "stacked brick" formation of bacteria attached to host cells when visualized using microscopy. *In vitro* models of adherence replicate the stacked-brick adherence pattern and a thick mucous biofilm (alternatively described as a mucous gel-like matrix) that is observed *in vivo*.[5] The criterion used to define EAEC – aggregative adherence – is observed in a large number of somewhat diverse strains of *E. coli*. Their common phenotype, though, suggests that adhesion proteins have an important role in their pathogenesis.

Recent research has shown that different strains of *E. coli* with the "stacked brick" aggregative phenotype have several different types of hydrophobic aggregative adherence fimbriae (proteins).[2] These include aggregative adherence fimbriae I (AAF/I, expressed by 31% of EAEC), AAF/II (expressed by 12% of EAEC), aggA, and aap.[8,5] However, some isolates do not have these genes, and therefore other proteins must mediate their adherence.[9,10] Toxins and virulence factors that are encoded on plasmids possessed by some of the EAEC strains include enteroaggregative heat-stable toxin 1 (EAST-1, present in 41% of EAEC strains, but also in 100% of O157:H7 enterohaemorrhagic *E. coli*, and 38% of normal flora *E. coli*), and a serine protease autotransporter protein (Pet, reported in 18 to 44% of EAEC isolates). A variety of other toxins produced by EAEC are also present in non-EAEC bacteria, including *Shigella* ssp, and *Yersinia* spp.

The gold standard method for EAEC identification is currently the HEp-2 adherence test. In this assay, colonies of *E. coli* obtained from stool cultures are inoculated in to monolayers of human epithelial (HEp-2) cells, and after three hours of incubation (culture), the cells are fixed, stained with crystal violet, and viewed using light microscopy to observed whether a "stacked-brick" pattern of adherent E. coli formed around the HEp-2 cells. This test requires specialized facilities, has a turn around time of several days, its interpretation can be subjective, and it is prone to contamination, and therefore it is not feasible for resource-limited settings.[11] Improvements to the test involving the use of HEp-2 cells that have been cultured, fixed, and dried ahead of time have made to render the test more feasible for a larger number of high-complexity labs, but these changes do not begin to make this test feasible for resource–limited settings.[11]

In another attempt to simplify the phenotype-based methods to detect EAEC, Wakimoto et al. developed an assay in which the biofilm generated by *E. coli* cultures in microplate wells was quantified using a microplate reader after staining with crystal violet. They determined that at a cut-off of > 0.2 at an OD570, the assay had a sensitivity of 100% and a specificity of 77.4%.[12] Though this assay requires fewer resources than the HEp-2 assay, it still requires culture of the stool specimen before performing the assay.

In summary, these phenotype-based tests are the current gold standard, and they identify a genetically diverse group of *E. coli* strains. Because approaches that require culture or microscopy require at least a moderate level of resources, they are not appropriate for resource-limited settings.

2B. Detection of EAEC DNA Biomarkers Using PCR Amplification Approaches

Methods for diagnosing EAEC that detect DNA sequences associated with the aggregative phenotype were developed in the early 1990s. One early approach, which detected a sequence sometimes referred to as CVD432 (now often called the aat gene) was initially reported to provide 89% sensitivity and 99% specificity for EAEC relative to the HEp-2 adherence assay.[13] Though later studies confirmed the high specificity, sensitivity was reported between 15% and 90%, depending on the region of the world.[5] Another assay that detects DNA sequences of the aggR gene has been published. This biomarker was shown to be very sensitive (i.e. identifying most strains that are positive in a phenotype assay), but much less specific (i.e. also identifying strains that do not have the aggregative phenotype). Other research groups reported initially promising performance by detecting other genes, but again this performance is not replicated in other studies.[14]

This theoretically promising approach (detecting EAEC by amplifying DNA sequences that are uniquely associated with the EAEC phenotype) has been hampered by the diversity in the bacterial strains that demonstrate the phenotype. Recent work by Jenkins et al. suggests that the genetic diversity is quite complex, and that there is no known DNA sequence which can identify all the *E. coli* isolates that demonstrate the aggregative phenotype in the HEp-2 assay. This group surveyed 143 isolates of "typical' and "atypical" EAEC strains for the presence of 13 DNA sequences (parts of genes), and concluded that the high heterogeneity among the strains makes it difficult to select a single gene as a biomarker that could be used to detect most isolates.[8] This study used a southern-hybridization based detection method, rather than a PCR amplification-based detection method, and observed that a significant fraction of strains from geographically diverse regions do not carry these genes at all. These genes, and other adhesin genes, are often carried on plasmids that are not universally present in all strains that demonstrate the adherent phenotype.[8] Therefore the low sensitivities of methods that detect these genes do not, thus far, appear to be the result of strain-to-strain sequence variation in these genes (which might adversely affect their ability to be amplified using PCR), but rather to their complete absence in a significant minority of strains. In recent years, many epidemiological studies have used these types of assays (for one or a few DNA sequences, in particular the aat gene) to detect EAEC, but most of these studies do not compare their performance to the HEp-2 or other phenotypic assay.[15,16,17,18,19] Given the sub-optimal sensitivity obtainable when amplifying the aat gene, this approach is not an ideal way to identify EAEC, but its wide-spread use is more likely a function of its simplicity (in resource-rich settings).

The results of this recent study by Jenkins et al. indicated that a multiplex assay that detected the aat, astA, and aaiA genes would have detected 134 of 143 isolates (94%) that were researcher-selected to provide a wide array of strain diversity, assuming no false negative results. It remains to be determined what the performance of such a multiplex PCR would be for detecting the strains with the adherent phenotype that are actually present in diverse geographical locations. Such a study should be designed to determine the effect of strain-to-strain sequence variation on the performance of this diagnostic approach.

In summary, a panel of EAEC-phenotype-associated genes would be required to provide adequate sensitivity and specificity for a PCR-based test with wide geographic applicability, and the evaluation of such a panel in clinical samples from patients served by resource-limited settings has yet to be performed. Therefore, the data required to determine if DNA sequence biomarkers could provide sufficient performance does not yet exist. DNA biomarkers have the potential advantage of being relatively sensitive, as well as a number of disadvantages that make them currently impractical for resource-limited settings, including a lack of commercially available products, the requirement for a high level of resources (including sample preparation), and their relatively high cost. In addition, approaches that use DNA amplification have the potential for cross-contamination of specimens.

2C. Detection of Parasites Using Microscopy Based Approaches

Until relatively recently, the diagnosis of giardiasis and cryptosporiasis required microscopic examination of stool specimens for the characteristic cyst and trophozoite forms of *G. lamblia*, and oocytes of *C. parvum*. This standard ovum-and-parasite (O&P) microscopy-based examination faces challenges even in resource-rich sites. The developmental stages, such as cysts or oocytes, that are detected by microscopy are not shed in the stool on a consistent basis from day to day after the acute infection

resolves, so that detection of infection using a single sample lacks sensitivity, and even studies that test two separate specimens report sensitivities ranging from 30 to 75%.[20,21,22] Regardless of its performance, high quality microscopy diagnosis is labor-intensive, requires significant training, can only be performed where electricity is available, is difficult to maintain in remote areas, and is therefore not practical for resource-limited settings.

2D. Detection of Parasite Antigens Using Immunodiagnostic Approaches

A variety of plate-based immunoassays and immuno-fluorescent staining techniques for microscopy are available for detecting *G. lamblia* and *C. parvum*,[22,23,21] but because of the high level of resources required for these methods, they are not appropriate for resource-limited settings.

Rapid diagnostic tests (RDTs) have been developed that utilize a lateral-flow immunochromatographic format to detect parasite-derived antigens in stool samples. In theory, this approach might provide better sensitivity than the visualization of parasites, because the levels of biomarker (antigen) in the stool may be reflective of many generations of parasites, not just those parasites of a particular stage in the lifecycle that are present in the stool at the time a sample is taken. This is an important issue for infectious agents that, in their chronic phases, are shed at low levels or intermittently into the stool, which hampers their detection using conventional O&P microscopy-based examination.[21] Parasite antigens that can be found in the stool and have been used as biomarkers are listed in Table 1.

Several individual-cassette-format immunodiagnostic products are commercially available to detect *G. lamblia* and *C. parvum* antigens in stool samples, including the Triage Parasite Panel (BioSite Diagnostics, San Diego, U.S.A.), the ColorPAC Giardia/Cryptosporidium (Becton Dickenson, Franklin Lakes, U.S.A.), and the CORIS *Giardia*-Strip and *Cryptosporidium*-Strip tests (CORIS Bioconcept, Gembloux, Belgium). The Triage kit requires 15 minutes to complete, and can accept fresh or freshly-frozen fecal samples. The sample is diluted, an aliquot is vortexted for 10 seconds, and centrifuged at 1,500 x g for 5 minutes. The supernatant is then poured into a sample filter device, and filtered into the filtrate tube. The sample must be filtered because the test device uses an alkaline phosphatase conjugate, and the enzyme alkaline phosphatase is also present in fecal material and the filtration step removes this enzyme. The test manufacturer believes that alkaline phosphatase conjugates provided the best sensitivity, and allow the test to be stored at room temperature, and therefore chose this enzyme for the detection reaction for those reasons. The filtered sample (0.5 mL) is then placed in the center of the test device (cassette). Enzyme conjugate is added to the device, and then six drops of wash solution are added in two consecutive steps. Then four drops of substrate are added, and allowed to incubate for 5 minutes at 15 to 25°C. Positive results are visualized as purple-black lines in the appropriate position in the results "window." All the tubes, pipettes, and reagents are included, as well as positive and negative control samples. This product also detects *Entamoeba histolytica* and *dispar*. The cost per test is about $ 20.[24]

The ColorPAC assay has a simpler workflow. The stool specimen (60 uL) is mixed with two drops of sample treatment buffer by pipetting. Then two drops of a capture antibody conjugate, and 2 drops of a detection reagent, are mixed into the tube as well. This mixture is poured into the test device, and results are read within 10 minutes. Positive results are visualized as grey-black lines in the appropriate positions in the "results window." All tubes, pipettes, devices and reagents are provided with the kit.[25] The

manufacturer indicates that this kit is extremely temperature sensitive, and must be kept at 2 to 8ºC at all times when not in use. All kit reagents, and any samples being tested, must be at room temperature (20 to 30ºC) prior to testing. The Giardia-Strip and Crypto-Strip products have a simple procedure as well (no centrifugation or filtration required), and use a colloidal gold conjugate for detection. The manufacture recommends this test kit be stored between 4 and 37°C.[26]

Studies available in the PubMed database that have examined the performance of these RDTs for the detection of *G. lamblia* and *C. parvum* are listed in Table 1. These studies were conducted in high-resource laboratories in relatively low-prevalence populations. Rows that present data generated using the same product are grouped by color.

Table 1. Parasite Antigen Biomarkers Currently Targeted by Rapid Diagnostic Tests

Publication, Test, Location	Species Detected	Antigen(s)	Specificity	Sensitivity
Garcia et al. 2000[20] Triage Parasite Panel Los Angeles, USA	*G. lamblia*	alpha-1-giardin[27]	95.9%[a]	97.4%[a]
	C. parvum	disulfide isomerase[28]	98.3%[a]	99.7%[a]
Sharp et al. 2001[24] Triage Parasite Panel Miami, USA	*G. lamblia*	alpha-1-giardin[27]	100%[a]	100%[a]
	C. parvum	disulfide isomerase[28]	100%[a]	99.8%[a]
Garcia et al. 2000[25] ColorPAC Los Angeles, USA	*G. lamblia*	cyst Wall Protein 1 (CWP1), also known as GSA-65[26]	100%[b]	100%[b]
	C. parvum	Not well characterized; believed to be a glycoprotein found on oocysts[26]	97.6%[b]	100%[b]
Katanik et al. 2001 ColorPAC Cleveland, USA	*G. lamblia*	cyst Wall Protein 1 (CWP1), also known as GSA-65[26]	100%[c]	100%[c]
	C. parvum	Not well characterized; believed to be a glycoprotein found on oocysts[26]	100%[c]	99.5%[c]
Oster et al. 2006[29] Giardia-Strip Heidelberg, Germany	*G. lamblia*	Not known; monoclonal antibody detects a cyst membrane antigen	58%[d]	99%[d]
Weitzel et al. 2006[30] *Giardia*-Strip and *Cryptosporidium*-Strip Berlin, Germany	*G. lamblia*	Not known; monoclonal antibody detects a cyst membrane antigen	44%[b]	≥98%[b]
	C. parvum	Not known; monoclonal antibody detects an antigen of the oocyte wall	75%[b]	≥98%[b]

a. Compared to ova and parasite (O&P) examination. Discrepant results were resolved using a plate-based immunoassay or immunofluorescence.
b. Compared to direct fluorescent-antibody assay, O&P examination, as well as plate-based immunoassays
c. Compared to ProSpecT Giardia/Cryptosporidium Microplate assay, and/or O&P examination
d. Compared to ProSpect Giardia-ELISA microplate assay (coproantigen test) (Remel, Lenexa, USA)

Another simplified lateral flow immunodiagnostic method for identifying infective pathogens that cause diarrhea was recently described by Dillman et al.[31] In this approach, which is specifically designed for

use in a resource-limited setting, a feces sample is diluted 100 to 1 and applied to a test cassette with a nitrocellulose membrane. Then a labeled antibody specific for the pathogen of interest is added, the membrane is washed, and a label-development substrate is added. Though this approach has been evaluated for a variety of diarrhea-causing infective agents, there are no published reports of its ability to detect EAEC, *C. parvum*, or *G. lamblia*.

In summary, relatively few studies have been published on the performance of these tests, and none have been conducted in moderate or low-resources settings, or in high prevalence populations. The available data indicates that the Triage and ColorPAC products have very high sensitivities and specificities, while the CORIS products have demonstrated unacceptably low sensitivities. The ColorPAC and CORIS products have the advantage of a relatively simple work flow, which does not require any equipment. While the cost of these products is generally low by diagnostic standards, the price of at least the Triage product is still very high for a resource-limited setting.

2E. Detection of C. parvum and G. lamblia DNA Using PCR-based Approaches

Assays to detect *C. parvum* and *G. lamblia* DNA have been developed and used in resource-rich settings, and at least one of these assays can distinguish among the different strains of these organisms.[19] There are no commercially available kits or systems for detecting these pathogens using nucleic acid amplification methods, so researchers have developed their own assays. These approaches have the advantage of providing the high sensitivity, and can be used to detect low-burden cases with very low parasite densities, that are often missed by the gold standard technique of microscopy. One approach has been reported to detect as low as 20 to 1.25 *G. lamblia* trophozoites per sample.[32] In this study on 97 specimens from patients with diarrhea in Bangladesh, India, a real-time PCR assay that amplified the 18s rRNA gene detected 95% (21 of 22) samples that were microscopy-positive, and 18% (13 of 74) that were microscopy negative for *G. lamblia*.[32] Because the samples that were microscopy-negative and PCR-positive had higher average cycle threshold values than the specimens that were positive by both approaches, the authors believe that these specimens represent true positives with very low-burden infections. Amar et al., using a multiplex PCR assay that detect the oocyst wall protein (COWP) gene and 18S rRNA gene of *C. parvum,* and the triose phosphate isomerase (tpi) gene of *G. lamblia*, also report that some of the patients in their study were positive for one sample, but not a second or third sample, suggesting that parasite DNA is also somewhat inconsistently distributed in the stool in some infections.[19] Because this study compared PCR amplification to microscopy performed (apparently) on a single sample, and did not include immunodiagnostic-based methods for these pathogens, it is difficult to conclude whether the PCR-positive but microscopy-negative samples were true or false positives. The authors of these studies did not evaluate the potential effects of strain-to-strain sequence variation on the performance of these DNA-based tests.

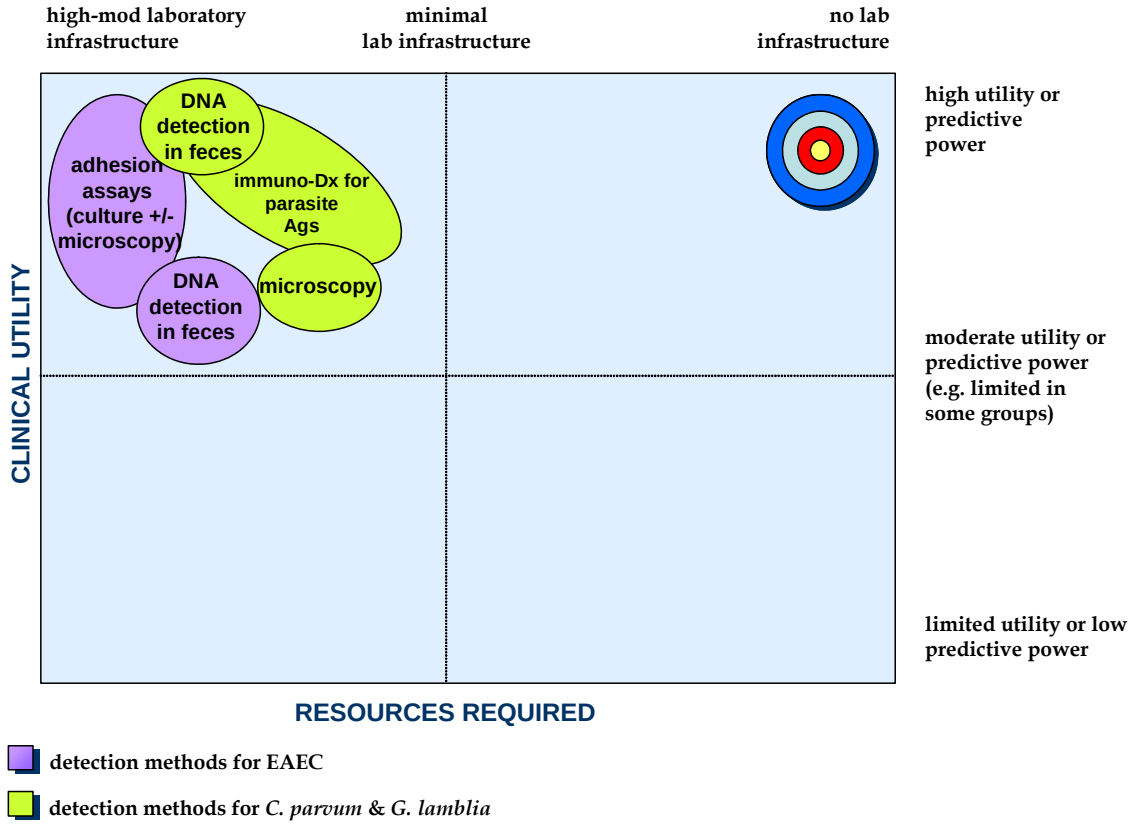
In summary, DNA biomarkers have the advantage of being a very sensitive method and their poor specificity is likely to be a reflection of the relatively insensitive method (microscopy) to which they are compared. The advantages that nucleic-acid based methods have in performance are accompanied by a number of disadvantages, including the lack of commercially available products, the requirement for a high level of resources, and their relatively high cost. In addition, they have the potential for cross-

contamination of specimens. Because of their current disadvantages, they are not appropriate for resource limited sites, though this picture is likely to change with time.

Summary of the Status of Biomarkers

The relative merits of the biomarkers and diagnostic approaches discussed in Section 2 are presented diagrammatically in Figure 1. In this figure, the ideal approach would be in the upper right quadrant, where the target is shown, because of its high predictive power (clinical utility in resource limited settings), and the low level of resources required for successful implementation. There are currently no approaches that can be performed in resource-limited settings. Approaches near the horizontal median line are limited by the biology of EAEC, *G. lamblia or C. parvum*, or of its human hosts. Approaches with high predictive power, but that currently require significant resources, are seen in the upper left quadrant.

Figure 1. Map of Currently Available Diagnostic Approaches for Diagnosis of EAEC, *C. parvum* and *G. lamblia* in Patients with Diarrhea



The positions of the spheres relative to the resources available in minimal-laboratory infrastructure settings are estimations, as there is no data on the performance of these approaches in such settings. The angle of the oval that represents the RDT immunodiagnostic devices indicates that some products require greater resources (such as a centrifuge or refrigerator), and that in settings with greater resources, greater clinical utility is likely to be achieved.

The only diagnostic approach that is anywhere close to being feasible for a resource-limited setting such as a health outpost is the RDT immunodiagnostic format. Though the commercially available RDTs have not been evaluated in resource-limited settings, it is likely that the very high performance of some products would be significantly affected by the storage and transport conditions that are often encountered in developing parts of the world. This is inferred from the manufacturer's recommendations for transport and storage under refrigerated conditions, as well as current perceptions regarding the causes of poor performance in resource-limited settings using RDTs for other diseases such as malaria and syphilis. The temperature stability requirements for these products, in addition to their relatively high cost, have kept these approaches to the left of the vertical median line in the figure.

3. Current Deficiencies in the Diagnosis of EAEC, *C. parvum*, and *G. lamblia* in Resource-Limited Settings

The deficiencies of the current approaches are summarized in Table 2. All of the methods listed use fecal samples, and therefore use a practical specimen type. Cells in the table that are filled in light blue are the characteristics that limit the utility of the biomarker or diagnostic approach in resource-limited settings.

Table 2. Summary of Current Deficiencies in Diagnostic Tests for EAEC, *C. parvum*, and G. *lamblia* in Patients with Diarrhea

Pathogen	Biomarker & Assay Format	TAT	Performance	Resources
EAEC	"stacked-brick" phenotype detected using HEp-2 adherence test	Days	The definitive gold standard. High sensitivity, high specificity	High
	Biofilm assay	Days	High sensitivity, high specificity	Mod-High
	DNA Detection using PCR-based approaches	Hours	Performance dependent upon which genes, and how many, are used. Adding genes generally improves specificity while lowering sensitivity.	High
C. parvum, G. lamblia	Visualization of parasites using microscopy	Minutes – 30 min	Moderate sensitivity. High specificity.	High
	Detection of parasite antigens using RDTs	Minutes	Some products demonstrate excellent sensitivity and specificity	Moderate (cold storage and shipping, cost)
	DNA sequences detected using PCR-based approaches	Hours	High sensitivity, high specificity	High

There is currently no single biomarker type and analytical approach that can use one assay on one sample to detect the three pathogens of interest in this document.

The diagnosis of EAEC infection remains challenging, even in resource-rich settings. The definitive phenotypic assays require culture and significant training, and therefore a high level of resources. Additional epidemiological information is required to determine how well a panel of DNA-based biomarkers might identify EAEC strains found in patients served by resource-limited settings in diverse geographical regions of the world. Approaches that detect DNA sequences lack either sensitivity or specificity, and also currently require a high level of resources.

For the diagnosis of *C. parvum*, and *G. lamblia* infection, the available biomarkers appear to be capable of providing excellent performance. Therefore the deficiencies that remain are related to the relatively high level of resources required for the commercially available tests. The first deficiency is likely to be the ability of the products to withstand the environmental challenges that are found in the transport and storage of diagnostic products to resource-limited settings, though there is currently no published data to indicate that they are actually unstable. A second deficiency is the lack of a low cost product that provides high performance.

Therefore, there are three major categories of deficiencies with the currently available biomarkers and diagnostic approaches available for detecting the pathogens of interest in this manuscript. These categories of deficiencies are presented in Table 3. Because the available methods for detecting *C. parvum* and *G. lamblia* are much closer to providing good performance in resource-limited settings, the majority of the issues listed in Table 3 deal with the diagnosis of EAEC.

Table 3. Categories of Deficiencies in Diagnostic Tests for EAEC, *C. parvum*, and G. *lamblia* to be Used in Resource-Limited Settings

Deficiency	Specific Issues for Particular Biomarkers and Approaches
No single biomarker type and analytical approach provides high performance in detecting all the pathogens of interest in a single sample	• Methods with good performance for EAEC and parasites use different biomarker types, and cannot be combined into a single assay. Two completely distinct assays would need to be performed. • DNA good for CP and GL; inadequate for EAEC • HEp-2 phenotype assay is unique for EAEC
The currently used biomarkers are or may be inadequate to fully inform the clinical decision	• Inadequate correlation between specific DNA sequences and the EAEC adherent phenotype
The resources required to perform the test are too high	• Adherence assays for EAEC (HEp-2 assay, biofilm assay) • Detection of DNA sequences: no commercially available products, no analytical platform that is ready for resource-limited settings, cost still relatively high. • Microscopy approaches require a high level of training and experience, and are not easily maintained in resource limited settings • Commercially available RDTs require a cool chain for storage and transport, and are still relatively expensive.

4. Opportunities to Improve the Clinical Performance of Existing Biomarkers

Many improvements could be made to existing test technologies that might allow the currently available biomarkers to deliver adequate performance in resource limited settings.

4A. Opportunity to Improve the Performance of DNA Sequence Biomarkers for EAEC

Many DNA sequences associated with the EAEC phenotype have been characterized in recent years, and the entire genome sequence for one EAEC strain has recently been completed.[3] It is possible that better performance could be achieved using DNA biomarkers by selecting a new or unique combination, and/or primers that amplify a wide variety of strains. Perhaps additional comparison of genomic sequences, and additional biological studies, will identify the genes associated with the atypical EAEC strains that use non-adhesin methods of adherence. New rapid sequencing technologies can be used to sequence multiple isolates of an organism's genome simultaneously and rapidly, within a few weeks. Such a study would reveal whether or not appropriate sequences exist. Probe and primer designs to permit the amplification of sequences that are relatively diverse have been successful on multiple occasions, and could solve the problem for EAEC detection.

4B. Opportunity to Implement a Field-Ready, Easy-to-Use Nucleic Acid-Based Test Platform

Though a field-ready, easy-to-use, and inexpensive platform to identify the parasite and EAEC DNA would be extremely useful, this is an extremely challenging undertaking that has been the goal of many groups for many years. In order to use DNA sequence biomarkers, a multiplex assay that distinguishes the three pathogens of interest would need to be adapted for a platform that could be used in resource-limited settings, which does not truly exist today. Platforms that are relatively close to meeting the requirements for a resource limited setting, such as the GeneXpert™ (Cepheid, Sunnyvale, U.S.A.) platform,[33] the LIAT™ (IQuum, Allston, U.S.A.) platform,[34] or the Handylab (Ann Arbor, U.S.A.) platform,[35] are likely to require additional engineering and manufacturing improvements, to reduce reagent and consumable costs, reduce manufacturing costs, reduce energy consumption, improve the robustness, improve the ability of local (perhaps less-specialized) technicians to make repairs in the country in which the test is being used, and to improve the ability of the reagents to withstand stability challenges throughout the supply chain.

4C. Opportunity to Improve the Stability and Affordability of RDTs That Detect Parasites

Though data on RDT product stability in resource-limited settings has not been published, it is likely to be an issue, based on the manufacturer's recommendations and experience with RDTs for other diseases, such as malaria, in resource-limited settings. It is widely believed that RDT products deteriorate under the shipping and storage conditions that are commonly found in the outlying areas of developing countries. Improvements to the binders, conjugation chemistries, dyes, and other components of the RDTs could address this issue. Though studies should be specifically designed to assess the stability of each of the components, it is easy to speculate that the antibodies used as binders are likely candidates for the most unstable part of the system. Synthetic binders, specifically selected for their ability to withstand high temperatures and other environmental challenges, might significantly improve the stability of RDTs, and once developed, might reduce their manufacturing cost and complexity. A variety of methods to develop synthetic binders have been developed, but in general, it has been difficult to replicate the specificity that can be obtained using antibodies. Recombinant antibody fragments (e.g. single chain antibodies) might provide an incremental improvement in stability, though a synthetic polymer or small molecule might ultimately be required to withstand 40°C for long periods of time. It is possible that a

company that has capabilities for the high-throughput use of combinatorial chemistry approaches to develop novel materials and surfaces, such as Symyx, might be able attempt an entirely new approach.

5. Evaluation of Known Molecules That Have Not Yet Been Clinically Validated

When considering the goal of developing a single diagnostic test that could identify all three pathogens, EAEC, *G. lamblia*, and *C. parvum*, the ideal test would detect biomarkers from all three pathogens in a single stool specimen. The assay format would need to provide high sensitivity and specificity for all three pathogens.

The antigen biomarkers currently detected by RDTs for *G. lamblia* and *C. parvum* already provide excellent performance, and therefore it is not recommended that additional biomarkers be evaluated, if an immunodiagnostic approach will be used for the final combined test that would be used to detect all three pathogens. The excellent performance of these antigen biomarkers for parasites makes it tempting to consider developing an immunoassay-based test for EAEC that could be added to an immuno-chromatographic RDT. There has been a tremendous amount of research into the biology of EAEC in recent years, and from this research a variety of options for antigen biomarkers of EAEC infection are now known. The potential for this approach is summarized in the following subsection.

5A. Evaluation of Known EAEC Protein Molecules as Biomarkers for Detection Using RDTs

It is intriguing to speculate that there might be an antigen, or (more likely) a number of EAEC antigens, that would be sufficiently specific for EAEC, and that could provide sufficient sensitivity when detected using an RDT format. An obvious target is the protein dispersin, encoded by the aspU locus. Dispersin is a secreted low-molecular weight protein that appears to coat the bacterial surface and prevent the adhesive fimbriae from adhering to the bacterial cell, and therefore leaves the fimbriae free to bind to the intestinal mucosa.[36] This protein appears to be present in the majority of EAEC strains from diverse geographical locations,[37] but this would need to be confirmed. Other options might be the adhesin molecules that EAEC use to aggregate, as they are secreted to the outside of the bacterial cell and are important to the aggregative phenotype.[5] AAF/I and AAF/II are perhaps the most well-characterized, and are expressed in about 40% of known strains, though it is unknown in what fraction of strains from developing countries.

Researchers continue to discover the adhesions used by other strains of EAEC, and recently Dudley et al. reported the characterization of a plasmid that encodes a novel set of adhesion molecules used by an atypical EAEC strain.[38] Work by Czeczulin et al. indicates that there are three major phylogenetic clusters of EAEC, which show a conserved linkage of virulence genes (including the adhesins) in each cluster.[37] Perhaps an antigen that is present in a high proportion of strains in each group could be identified and used to detect EAEC via immunochromatographic methods.

It is still unknown whether any of these proteins are found in consistently detectable levels in the stool. If one or more of these molecules were found to be present in high enough levels, then binders would need to be developed for these molecules.

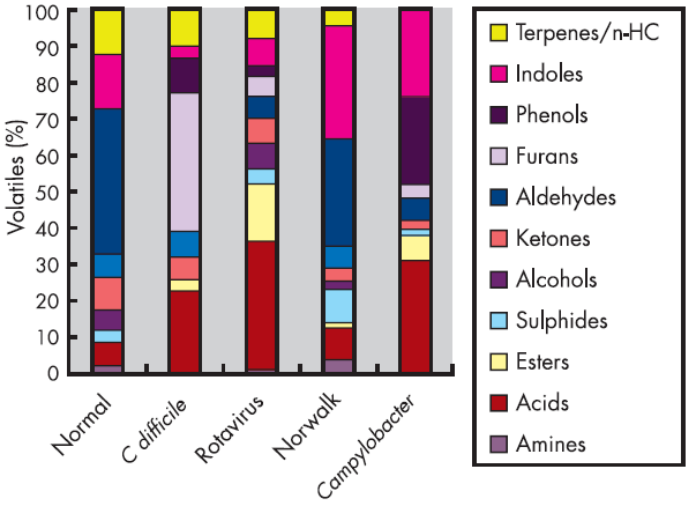
Another approach would be to clone, express, and raise antibodies against bacterial proteins with the intent of screening samples to determine which, if any, of the proteins is a candidate biomarker with sufficient sensitivity and specificity. This approach might identify protein antigens, or a combination of protein antigens, that are not currently known or well-characterized.

6. Approaches for the Discovery of Novel Biomarkers for Diagnosis of EAEC, *C. parvum* and *G. lamblia*

The category of biomarkers for which there is very little data are the volatile organic compounds (VOCs) released from stool samples. This intriguing and underexploited category of biomarkers is discussed in the following section.

6A. Discovery of Volatile Organic Biomarkers

Studies conducted more than 20 years ago produced evidence that the metabolism of many pathogens that cause diarrhea may be sufficiently different to allow their identification via chemicals (primarily metabolites) found in the feces.[39] Other research programs that have investigated the biology of *G. lamblia* have determined that this parasite, like many others, has a unique metabolism,[6] which may result in the production of a VOC, or quantities of particular VOCs, in the feces which could be identifying for particular pathogens. One study by Probert et al. specifically investigated the possibility of using VOCs detected in the vapor (or "headspace") of diarrheagic stools. In this study, stool samples from 35 patients with infectious diarrhea, and from 6 healthy controls, were analyzed using a gas chromatography – mass spectrometry (GC-MS) analytical technique. The stool samples (approximately 0.75 g) were placed in 10 mL sealed headspace vials designed for solid phase microextraction (SPEM). The gas within the headspace was then analyzed using GC-MS by absorbing the VOCs from the headspace onto the SPME fiber. The VOCs were then desorbed from the fiber thermally via splitless injection onto a thin film column. VOCs were eluted from the column directly into a small, benchtop quadrupole MS instrument, the Hewlett Packard 5971. This study found that the compounds in stools from healthy individuals were remarkably similar, but that those from individuals with diarrhea caused by specific etiologies were consistently different, and that



Metabolic profile of volatile classes from stool samples of normal subjects and from patients with infectious diarrheas (average of six samples for each sample type).[40]

characteristic changes in the VOC pattern occurred with specific types of diarrhea.[40] The authors looked at the predictive power of individual VOCs, rather than attempting to identify a pattern that distinguishes with high predictive power. Unfortunately this publication did not include data from patients with EAEC, *C. parvum* or *G. lamblia* infection. These studies remain to be conducted. It is not difficult to envision that the challenge for this approach will be distinguishing EAEC from the *E. coli* strains that normally inhabit the human gut, and from the other types of pathogenic *E. coli* strains.

In an experimental program designed to discover VOC biomarker patterns, a hypothesis-free approach could be undertaken which would involve an analysis of the VOCs emitted from stool specimens using either electronic nose or mass spectrometry instruments. Devices characterized as electronic noses (e-noses) utilize a variety of technologies, though perhaps the most prevalent utilizes an array of non-specific chemical sensors that bind volatile chemicals in the vapor headspace over a sample. The most common types of sensors are metal oxide sensors, conducting polymers, and piezoelectric-based sensors. The sensors typically have a partial specificity, in that they respond to certain classes of chemicals, such as alcohols and aldehydes, rather than to single compounds. The interaction of volatile compounds with the sensor surface results in changes in the physical properties of the sensor, such as its resistance, conductivity, and frequency, which are then measured. Therefore the nature and relative ratio of the molecules in the headspace determines the response pattern of the sensor array. The device is used to sample a set of "case" samples (e.g., known cases of diarrheal caused by *G. lamblia*) and control samples (e.g., patients with diarrhea caused by other pathogens, patients with other illnesses, and healthy individuals), and pattern-matching algorithms are used to identify a pattern that segregates the cases from the controls. The device can then use the pattern to classify unknown samples. At least one commercially available e-nose model is portable and battery powered. The Cyranose 320, developed by Cyrano Sciences but now sold by Smiths Detection, is a hand-held, battery-powered device designed for field-use. One drawback to the e-nose approach is that molecular identity of the molecules that make up the pattern cannot be determined, because the device only recognizes the pattern.

Another hypothesis-free approach to discovering biomarkers from stool specimens utilizes mass spectrometry detection methods, which can often identify the particular VOCs that are unique to a disease etiology. An experimental program would therefore need to be undertaken, to determine if VOCs that are unique to the three pathogens under consideration could be detected as they are emitted from stool samples of patients with diarrhea. This initial program could utilize either MS or an e-nose detection approach. It is possible that if the important principle components of the VOCs for each pathogen are identified via MS approaches, then an e-nose instrument (of which at least one is already field-deployable) could be specifically designed to detect and discriminate the important molecules.

The Cyranose 320[41]

7. Clinical Sample and Study Design Issues for Biomarker Discovery and Validation

Initial studies that evaluate the potential of EAEC antigens could be conducted in a resource-rich sites, using fresh specimens from local children with diarrhea, and perhaps evaluating banked cultured strains of EAEC from around the world (for instance, see strain collections in Jenkins et al.[8] and Czeczulin et al.[37]) Because little is known about prevalence of different EAEC strains in resource-limited sites, a relatively large collection of strains from children with chronic diarrhea in resource-limited settings around the world would need to be undertaken, to ensure that most strains can be detected by any test that is developed. Because EAEC is likely to cause only a fraction of the chronic diarrhea, thousands of specimens might need to be collected in any one location, in order to assure that dozens or hundreds of EAEC strains are found and that the collected specimens accurately represent the proportions of endemic strains. A pilot study involving a smaller number samples could be conducted first. The challenges of culturing all these specimens in a local lab, in order to perform a phenotype-based assay which will be used to compare to the new method, could be significant.

Stool specimens from which volatile organics might be measured will need to be collected prospectively. These initial discovery studies could be conducted in a group of children (perhaps low hundreds) with diarrhea caused by the three pathogens of interest in this document, as well as diarrhea caused by other pathogens, children with other conditions such as malnutrition, and healthy children. If this approach looks promising, then additional validation studies would need to be conducted with patients from resource-limited settings.. If samples (both VOC and fresh stool specimens) could be transported in some way to a diagnostic lab, this would make the later rounds of validation much easier. Initial exploration of a VOC approach should therefore include studies to determine if samples can be stored and transported..

8. Discussion and Recommendations for the Improvement of Diagnostics for Diarrheal Disease Pathogens

The following recommendations are presented for consideration, given the deficiencies of current diagnostic test methods for deployment in resource-limited settings, the opportunities for improving the performance of existing biomarkers, and the approaches that might be used for discovering novel biomarkers that are more appropriate for use in resource-limited settings.

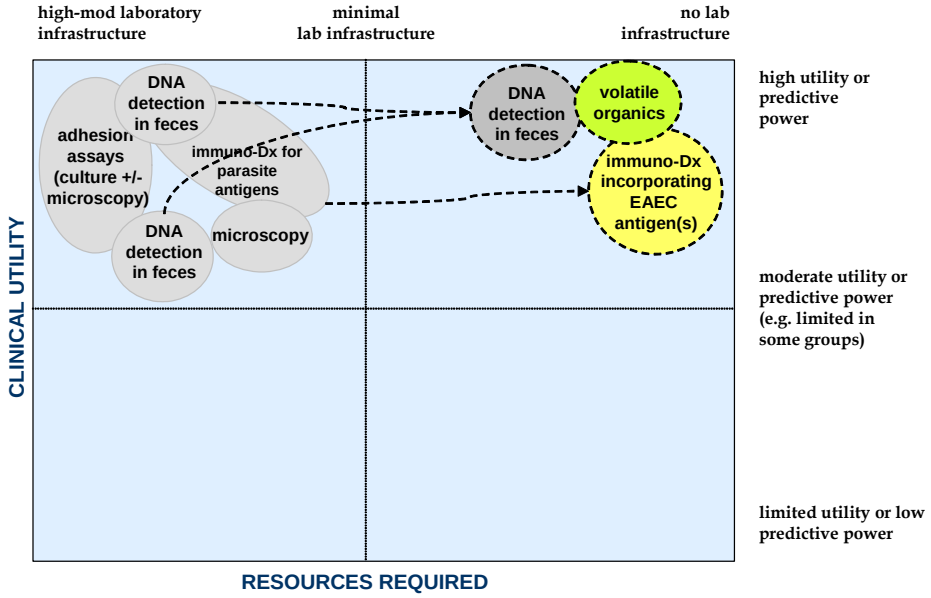
8A. What clinical information and user specifications are required for the design and development of the diagnostic products needed for the detection of the selected diarrheal pathogens?

For identifying EAEC, *C. parvum* and *G. lamblia* in patients with diarrhea, the ideal diagnostic test would provide good sensitivity and specificity for all three pathogens in a single assay using a single specimen. The assay should distinguish all the pathogens so that appropriate treatment could be administered. In particular, the test should be able to detect the lower levels of pathogens that may be present in the chronic stages of diarrhea when fewer pathogens are shed into the stool. The test products must withstand the rigors of the transport and storage chain that are likely to be encountered. The test procedure must be simple enough to be performed by a health care worker in a health outpost setting and inexpensive enough to be practical for resource-limited settings.

8B. What biomarkers, sample types, and technologies are most appropriate for 3 diarrheal pathogens?

For detecting EAEC, the existing biomarkers with the best clinical performance are not practical for resource-limited settings and are unlikely to ever be so. DNA biomarkers do not currently provide adequate performance, but additional research may identify better DNA biomarkers. In any case, at this time, a relatively high level of resources is currently required to detect DNA biomarkers. Detection of pathogen-derived antigens in a rapid immunodiagnostic format could be very simple enough, if it is shown that EAEC can be detected this way. The use of VOCs appears promising, but the challenge is likely to be the specificity for EAEC. Detection of VOCs using an e-nose is intriguing because many pathogens could be identified simultaneously, no reagents and few consumables are required, and a field-deployable instrument is already available. These future diagnostic approaches are depicted in Figure 2. In this Figure, current approaches are shown as light gray spheres and future approaches are shown as colored spheres. The position of each sphere illustrates the resource requirements (x-axis) and the clinical utility of the test in resource-limited settings (y-axis). The ideal biomarker and test method would be in the upper right quadrant, with a high predictive power and low resource requirements. The vertical placement of methods in this figure is speculative, as there is little data on the clinical utility of these tests.

Figure 2. Future Approaches for a Diagnostic Test for 3 Pathogens in Patients with Chronic Diarrhea



8C. Recommended Course of Action and Resources Required

There are three potential paths forward for the development of a diagnostic test for EAEC, *C. parvum* and *G. lamblia* that are appropriate for resource-limited settings and are discussed in the following sections.

Potential Path Forward 1: Identify antigen biomarkers for EAEC, to be added to an existing RDT test, and modify existing parasite RDTs to improve stability and affordability.

In order to develop a rapid immunodiagnostic product, two initial milestones must be achieved. First, it must be determined whether there are antigen(s) of EAEC in the stool that will allow it to be sensitively and specifically detected using a rapid, point of care immunochromatographic format. Binders that allow the detection of these additional biomarkers would need to be combined with methods to detect parasite antigens in a single immunochromatographic device. Then, a variety of evaluations of the existing parasite RDTs should be undertaken by a group that is very experienced in the development of point of care immunodiagnostic tests. The thermostability of the components of existing tests, and their stability in the face of other environmental challenges should be systematically evaluated; and a plan should be devised to develop alternative components for those that are not sufficiently stable. Such improvements might include changes to the detection chemistry or specific binders. This path forward has a moderate degree of biological risk and a moderate degree of technical risk. The biological risk is moderate because it is unknown if EAEC infection produces sufficient quantities of fairly specific antigens that can be detected in the stool, but there are a number of promising candidates that are already known. The technology risk is moderate since at least a triplex assay would need to be developed and the immunochromatographic devices would need to be re-designed to lower the resources required.

Potential Path Forward 2: Identify volatile organic compounds from stool samples, to be detected using an electronic nose

The discovery and validation of volatile organic biomarkers for the diagnosis of diarrhea-causing pathogens is intriguing, given the small amount of preliminary evidence that the VOCs produced by stool samples contain a great deal of information. Given that the analytical technology already exists for discovery (e.g., an e-nose, or mass spectrometry) it should be possible to complete an initial evaluation in a lab that already has the capability. This experimental program would then need to be repeated in populations that are served by resource-limited settings, which would require prospectively-collected samples. If the biomarkers were validated, then it is likely that an engineering program would need to be initiated to improve the affordability and robustness of the analytical instrument such as an e-nose. This would require additional time relative to existing methods, and the cost-benefits of waiting for this more robust and affordable instrument could be weighed against the option of distributing an existing, more costly, and perhaps less-robust model immediately.

The proposed experimental program has a moderate degree of both biological and technical risk. The biological risk is moderate because although there is already evidence suggesting that many pathogens can be distinguished in the stool, the ability to distinguish EAEC from other pathogenic forms of *E. coli*, and non-pathogenic *E. coli* found in the gut might be difficult to achieve. The technology risk is moderate because it is still unclear that the existing e-nose instruments have an adequate set of sensors for the detection of the important molecules present in a clinical sample;, yet, there are already commercially available, portable MCC-IMS and e-nose instruments that could be used in the evaluation studies, and which might be migrated to a truly field-deployable commercialization platform. If useful biomarkers were identified using mass spectrometry approaches, then perhaps the knowledge of the specific molecules to be detected could be used to fine-tune the array of sensors in an electronic nose, which might result in a better field-usable instrument.

Potential Path Forward 3: Identify a DNA-based biomarker with high performance for EAEC, and develop a field-deployable platform for detection of DNA-based biomarkers

The ability to use DNA-based biomarkers will be limited by two factors: 1) the identification of a multiplex assay that sensitively and specifically detects EAEC, and 2) the development of a platform for detecting DNA-based biomarkers that is useful in resource-limited settings. The challenges involved in implementing this type of platform in resource-limited settings are significant. As opposed to the other options presented, sample purification and a supply chain for reagents would be required for DNA testing as it is performed today. If these other options (outlined in Paths 1 and 2) are incapable of providing the necessary clinical performance, then DNA sequences detected using a nucleic acid testing platform would be the next option. In order to detect DNA biomarkers, improvements to the ease-of-use, robustness, affordability of the instrument, affordability of the reagents and consumables, and probably the stability of the reagents, would all be required.

This path forward has a moderate degree of biological risk and a high degree of technical risk. The biological risk is moderate, because efforts to identify a set of DNA sequences that are both sensitive and specific for EAEC have not been successful. The technology risk is high because a significant decrease in the local resources required would need to be achieved in even the existing platforms (e.g. GeneXpert or LIAT), including the platform robustness, power required, ease-of-use, and cost. It is also possible that improvement to the reagents would be required to enhance their stability throughout the supply and storage chain.

Summary of Recommendations

For the diagnosis of EAEC, *C. parvum* and *G. lamblia* infection in children with chronic diarrhea, the development of a rapid immunochromatographic device that can simultaneously detect all three pathogens is the most straightforward and least-risky path forward , given the very low resources required to perform this diagnostic approach, and the potential for antigen biomarkers to provide adequate performance. The use of VOCs is also intriguing, and the initial studies focusing on the most biologically-challenging question - whether EAEC could be distinguished from other pathogenic and non-pathogenic *E. coli* - could be conducted either in parallel with the efforts to develop a new rapid immunochromatographic device, or delayed until a point in time when it becomes clear whether volatile organics detected using e-nose approaches appear likely for another indication. Once the proof-of-concept experiments are completed for the immunodiagnostic and volatile organic pathways, then the most promising approach could be prioritized. If neither of these approaches appears biologically feasible, then the third path – detection of DNA sequences – could be pursued, which is likely to be the most technically challenging of the options that have been presented.

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