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Molecular Diagnosis of Diarrhea: Current Status and Future Potential

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Abstract Determining the microbiologic etiology of enteric infection remains an elusive goal. Conventional approaches, including culture, microscopy, and antigen-based tests have significant limitations such as limit of detection and the need for multiple procedures. Molecular diagnostics, especially PCR based tests, are rapidly changing research and practice in infectious diseases. Diarrheal disease, with its broad range of potential infectious etiologies, is well suited for multiplex molecular testing. This review highlights examples of currently employed molecular tests, as well as ways in which these tests can be applied in the future. The absence of a gold standard for the microbiologic cause of diarrhea means that the clinical significance of detected organisms may not always be clear. Conventional wisdom is that there should be one main pathogen causing diarrhea, however our thinking is challenged by increased detection of mixed infections. Thus, the successful incorporation of molecular diagnostics for diarrheal disease into practice will require both a careful understanding of the technical aspects and research to define their clinical utility.

Keywords Diarrhea · Molecular diagnosis · PCR · Enteropathogens

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Introduction

Diarrhea is a disease of high mortality and morbidity in both endemic and epidemic settings [1]. The number of etiologic agents implicated in diarrheal disease is vast and includes viruses, bacteria, protozoa, and helminths [2]. The principle diagnostic modalities have historically included culture, microscopy, and antigen-based tests. Culture methods are often low yield for enteropathogens, particularly in the setting of antibiotic use [3]. Microscopy for parasites is also insensitive and, although inexpensive, requires substantial time, equipment, and training. Antigen detection tests on stool have proliferated since the 1970s and have been a major advance for diarrheal diagnostics [4]. However antigen detection assays are costly and exist for only a limited number of pathogens (Table 1), namely rotavirus, adenovirus, astrovirus, norovirus, Campylobacter, Clostridium difficile, Giardia, Cryptosporidium, and Entamoeba [5].

Molecular diagnostics have an emerging role in the diagnosis of infectious diseases. These tests generally involve the amplification of DNA or RNA, with PCR the most common amplification strategy. US FDA-approved nucleic acid amplification tests exist for tuberculosis, gonorrhea, chlamydia, and many viral infectious including HSV, CMV, Hepatitis B and C, HIV. This can be a strength by offering increased sensitivity. Indeed these molecular tests have consistently demonstrated excellent sensitivity when compared with traditional diagnostics. There is great promise for molecular diagnostics for diarrhea in particular, where enhanced sensitivity is desirable since diagnostic yield is typically poor. Sensitive detection of DNA or RNA can also be a hindrance, if one detects low and clinically insignificant levels pathogens. It goes without saying that detection of DNA or RNA does not denote infectious particles or viability. This review attempts to both describe

Table 1 Enteropathogens, available diagnostic modalities, and molecular targets

Enteropathogens	Commonly available diagnostic modalities	Example of targets available for molecular testing	Clinical comments
Viral			
Rotavirus	Antigen detection	NSP3	A major cause of severe diarrhea in 6–24 month children
Norovirus	Antigen detection (insensitive vs. RT-PCR)	ORF1-ORF2	Important cause of outbreaks and community diarrhea
Adenovirus	Antigen detection	Hexon	Serotypes 40/41 are most commonly reported to cause diarrhea
Astrovirus	Antigen detection	Capsid	
Sapovirus	RT-PCR (rarely pursued)	RdRp-capsid	
Mimiviruses, Aichivirus, Bocavirus, Parechovirus, Cytomegalovirus, Hepatitis A, Coronaviruses, Picornaviruses, Toroviruses, other enteroviruses	Rarely pursued, but could involve RT-PCR or culture	Diverse	Rare or uncertain viral causes
Bacteria			
Clostridium difficile	Antigen detection or PCR	tcdA, tcdB	Hospitalized and antibiotic-exposed patients
Enterotoxigenic E. coli	Culture with genotyping or toxin assay of colonies	LT, ST	A major cause of diarrhea in children and adults worldwide
Enteroaggregative E. coli	Culture with genotyping or tissue culture cell assay of colonies	Diverse	Emerging cause of diarrhea
Enterohemorrhagic E. coli	Shiga toxin detection of enrichment broths or culture	Stx1, Stx2	Important cause of bloody diarrhea and HUS, associated with undercooked meat
Enteropathogenic E. coli	Culture with genotyping or tissue culture cell assay of colonies	bfpA, Eae	Severe diarrhea in infants, Reports of hospital outbreaks
Enteroinvasive E. coli	Culture	Ial/ipaH	Sporadic food borne outbreaks
Shigella	Culture	Ial/ipaH	Major cause of acute diarrhea and dystentery
Salmonella	Culture	invA	Important foodborne pathogen in developed and developing countries
Campylobacter	Antigen detection or culture	cadF	Often associated with diarrhea in infants, then colonization becomes common in developing countries
Aeromonas	Culture	Aerolysin	
Vibrio cholera and parahaemolyticus	Culture	toxR	Outbreaks and seen in older children/adults
Yersinia	Culture	lysP	
Listeria	Culture	*iap*	
Toxigenic B. fragilis Tropheryma whippelii, Anaerobiospirillum, Mycobacterium spp.	Culture, histopathology	Varies	Rare or uncertain bacterial causes
Protozoa			
Cryptosporidium spp.	Microscopy with modified acid fast stain or antigen detection	COWP	Major cause of both acute and persistent diarrhea
Giardia lamblia	Microscopy or antigen detection	18S rRNA	High carriage rates in developing countries makes clinical significance unclear
Entamoeba histolytica	Microscopy or antigen detection	18S rRNA	Cause of diarrhea and dysentery
Cyclospora cayetanensis	Microscopy with modified acid fast stain	18S rRNA	
Cystoisospora belli	Microscopy with modified acid fast stain	5.8s & ITS2	
Dientamoeba fragilis	Microscopy	18S rRNA, other	Rare or uncertain protozoal causes

Table 1 (continued)

Enteropathogens	Commonly available diagnostic modalities	Example of targets available for molecular testing	Clinical comments
Balantidium coli			
Blastocystis hominis			
Helminths			Generally rare causes of diarrhea
Ascaris lumbricoides	Microscopy	ITS1	
Necator americanus	Microscopy	ITS2	
Ancylostoma duodenale	Microscopy	ITS2	
Strongyloides stercoralis	Microscopy	18S rRNA	
Trichuris trichiura	Microscopy	18S rRNA	
Schistosoma spp, Trichinella	Microscopy	18S rRNA	Rare or uncertain helminthic causes
Fungal			
Enterocytozoon bieneusi	Microscopy with modified trichrome stain	18S rRNA	
Encephalitozoon intestinalis	Microscopy with modified trichrome stain	18S rRNA	
Candida albicans, Histoplasma, Blastocystis	Microscopy, culture	ITS2, other	Rare or uncertain fungal causes

the wide variety of molecular strategies available and under development for enteropathogens and discuss the clinical scenarios where these strategies might be of value.

Molecular Diagnostic Strategies

Nucleic Acid Extraction

The first step in any molecular diagnostic procedure is extraction of nucleic acid from the specimen. In the context of diarrhea the specimen is usually direct stool, however stool cultures or broths are often used for bacteria. The extraction step is important, since stool is a complex mixture rich in diverse nucleic acids and amplification inhibitors. In our hands, detection of a given target is often several logs reduced when placed in a stool mixture. Stool cultures or broths are inherently less complex and target genes will have multiplied by virtue of culture, which is advantageous, however this culture of course introduces an additional step.

Singleplex vs. Multiplex PCR

Singleplex PCR utilizes a single set of primers to amplify one target at a time. Detection of the amplified target can occur by several means, including gel-based electrophoresis or fluorescent reporter molecules, most often using sequence-specific probes. In PCR employing these probes, or using intercalating dyes, fluorescence can be interrogated after every amplification cycle and this method is thus termed “real-time” PCR. PCR has been used for years and usually offers greater sensitivity than conventional methods. For instance, in a study of 127 stool samples from patients with symptoms of acute gastroenteritis, 18 were culture positive for *Campylobacter* whereas 58 were PCR positive [6]. An investigation of real-time PCR detection of microsporidia demonstrated a lower limit of detection of 10^2 spores/mL stool, versus 10^6 spores/mL for microscopy [7]. In a large study of clinical samples, Amar et al. employed PCR on stool to re-examine the English case–control Infectious Intestinal Disease Study [8••]. PCR increased the enteropathogen detection rate from 53% to 75% of cases as well as from 19% to 42% in controls. The detection rate increased for both viral and bacterial enteropathogens and, not surprisingly, the number of samples with multiple pathogens detected increased. Therefore, while the potential for increased diagnostic yield is substantial, the clinical significance of isolated PCR findings can become less clear.

The wide variety of potential pathogens that can be associated with diarrhea make the use of singleplex PCR unwieldy for syndromic testing. Indeed one can enumerate over 50 pathogens that could be implicated as causing diarrhea (Table 1). Multiplex PCR denotes the amplification of multiple targets in a single reaction. Discrimination of distinct targets requires sequence-specific probes, size differences of the DNA amplicons by gel analysis [9, 10], or by examining the melting characteristics of amplicons [11, 12]. Our group has used multiplex PCR reactions using Luminex beads for detection as a means to increase the utility of multiplex testing [13, 14]. The future will see

more multi-target amplification tests to offer syndromic testing for diarrheal pathogens. These will include closed multiplexed and arrayed singleplex systems, which have been recently employed to determine the etiology of respiratory infections [15, 16]. A multi-target test for enteropathogens has recently been developed by Luminex and approved for use in Europe (xTAG GPP[17]). The same technology has been used to serotype Shiga toxin-producing *Escherichia coli* isolates[18].

Quantitative PCR

Molecular strategies, though highly sensitive, may result in the detection of low levels of enteropathogens with unclear clinical significance. This is particularly vexing in developing countries where certain enteropathogens, such as *Giardia* [19] and many viruses, are known to occur at high rates even in individuals without diarrhea, raising the question of what is a pathogen. Ultimately approaches that can offer quantitative detection may prove useful to infer clinical significance. The underlying assumption is that pathogens present at high burden are more likely to be associated with disease. Quantitative detection is implicit to real-time PCR, where the cycle time to positivity is recorded by the cycler. Further refinements to quantitation include use of standard curves of known quantities of targets, and use of spiked controls that control for sample-to-sample variability in nucleic acid extraction and amplification efficiency. Phillips et al. have applied these approaches towards rotavirus and norovirus [20, 21••]. Quantitative techniques leverage the speed and sensitivity of PCR while also potentially offering clinical relevance. However this will require much more clinical evaluation since a quantitative relationship between pathogen burden and symptoms is not known to exist for many pathogens, and need not necessarily be the case for all individuals.

Incorporation of Molecular Tests into Diagnostic Algorithms

In some scenarios a combination of conventional and molecular methods can be effective. For example, PCR can be used as a high-sensitivity screening test to determine a subset of samples that warrant conventional testing. A study by de Boer et al. described such a molecular screening approach for the detection of five major enteric pathogens [22•]. In an analysis of 28,185 stool samples received for detection of bacterial and/or parasitic enteropathogens, the algorithm including molecular screening significantly decreased the testing burden for a clinical microbiology laboratory over the course of a 2-year period. At the same time the strategy led to an increase in the total pathogen direction rate over conventional methods from 6.4% to 19.6%, and a greater rate of detection of multiple pathogens. In this setting one could envision using molecular assays to rule cases out, conventional methods to rule cases in, while molecular positive/conventional negative samples would need further evaluation or confirmation. Similar approaches can be employed in epidemiologic studies. Gladstone et al. investigated the protective effect of natural rotavirus infection in a birth cohort [23]. Here, both surveillance (non-diarrheal) stool samples and diarrheal samples were tested for rotavirus with both an ELISA and PCR assay. Samples were considered positive if either two ELISA tests or one PCR test was positive.

Low-Cost Molecular Diagnostics

While PCR tests can offer timely recognition of cases, currently they are primarily confined to resource-rich centralized laboratories. There is a pressing need to improve universal access to effective diarrheal disease diagnostics [24]. Ricci et al. suggested that a highly-sensitive diagnostic for *Giardia lamblia*, *Cryptosporidium parvum*, and enteroaggregative *Escherichia* coli could reduce stunting and its long-term consequences by 12.5% in children with diarrheal illness [25]. Yet the challenge is how to provide sophisticated molecular diagnostics to a setting with limited infrastructure. The ideal test would be affordable, easy to use, rapid, refrigeration-free, transportable, and offer good performance [26]. Microfluidic technologies with miniaturization of PCR can allow for faster tests, lower reagent costs, and portability [27]. LaBarre et al. recently described a prototype of electricity-free, isothermal nucleic acid amplification strategy [28]. This is an area of active research and development.

Clostridium Difficile-Associated Disease (CDAD)

Due to the increased incidence and virulence of CDAD, there has been substantial interest in improving existing diagnostic modalities. Tests for CDAD have historically fall into two main categories: those which culture the organism directly and those which assess for presence of the associated toxin. Stool culture and the cell culture neutralization assay (CCNA), the established gold standard tests, are now rarely employed clinically [29]. Enzyme immunoassays (EIAs) for toxin detection remain the most frequently used tests, but lack sensitivity when compared to anaerobic stool culture. A comparison of nine commercially available EIAs yielded an average sensitivity and specificity of 82.8% and 95.4%, respectively [30]. The test characteristics of toxin-based tests also appear to vary significantly by *C. difficile* strain type [31]. Recently, PCR-based tests on stool targeting the toxin B gene have been adopted by some institutions for use either as a stand-alone test or as confirmation after a high-

sensitivity screening test. Swindells et al. compared two commercial PCR tests for toxin B (GeneOhm PCR and Xpert *C. difficile* PCR) to stool culture in 150 consecutive liquid stool specimens from hospitalized patients [32]. When compared to toxigenic stool culture, they had a sensitivity and specificity of 100% and 97% and 100% and 99.2%, respectively. A second study evaluated the GeneOhm PCR test in comparison to CCNA and toxigenic stool culture in 377 symptomatic patients with a mix of liquid and soft stools [29] which revealed a sensitivity of only 83.6% and 98.2%, respectively. At present many institutions empirically isolate patients with clinical suspicion for *C. difficile* while awaiting test results, and PCR-based algorithms stand to improve this triaging system [33]. That said, clinical suspicion remains paramount in the era of molecular diagnostics, since indiscriminate use high sensitivity diagnostics may uncover positive results of unclear significance and lead to overuse of antibiotics.

Epidemic Shiga Toxin-Producing *Escherichia coli* (STEC)

Molecular diagnostics were a prominent part of the rapid identification of an outbreak of a novel strain of STEC in May 2011. Molecular diagnostics allowed for rapid and specific characterization of the nature and extent of the outbreak. They identified the novel hybridization of virulence factors while suggesting that the outbreak may not have been zoonotic in origin. Specifically, Frank et al. used PCR tests to screen for both Shiga toxin–producing *E. coli* virulence-factor genes as well as genes typical of enteroaggregative *E. coli* [34]. Bielaszewska et al. utilized the molecular analysis of an early isolate to develop a multiplex PCR specific to the strain, which was then used for ongoing identification of outbreak isolates [35].

Molecular Diagnostics and Antibiotic Susceptibility

In an era of increasing antibiotic resistance, molecular diagnostics are being developed to ascertain antibiotic susceptibility. Phenotypic antibiotic susceptibility testing relies on the assessment of in vitro growth in presence of antibiotics. Molecular diagnostics offer a more targeted result of known genetic markers of resistance. Examples include detection of the mecA gene by PCR for detection of methicillin-resistant *Staphylococcus aureus* [36], and mutations in the rpoB gene are reliable indicators of rifampin susceptibility in Tuberculosis [37]. In the context of diarrhea, it would be desirable to couple molecular detection of bacterial pathogens such as Shigella, Salmonella, and Campylobacter with resistance testing since resistance is common and increasing [38]. Detection of resistance markers in stool DNA extracts will be inherently challenging or impossible because of the diversity of organisms and sequences in stool, and thus inability to ascribe detection to a particular pathogen. However, these methods could be used on pure cultures (e.g., gyrA/B mutations to detect quinolone resistance in Shigella). This is another example of the power of combining conventional and molecular approaches.

Conclusions

Molecular diagnostics are here to stay and will enter the world of enteric infections for the foreseeable future. However, it is important to restate several caveats that will impact our use of molecular diagnostics in the context of diarrhea. First, the high rates of asymptomatic carriage of enteropathogens, particularly in developing countries, will make it difficult to determine the clinical significance to certain isolated organisms with risk of overdiagnosis. Here we think quantitation may be useful, and treatment algorithms based on these tests with increased sensitivity will require clinical validation. Second, the costs associated with these tests are significant (e.g., tens of dollars per test). In a setting in which the primary treatment modality is often supportive care and oral rehydration, the clinical contexts in which molecular diagnosis will be of value will need to be defined. These might include scenarios where rapid diagnosis may help target public health interventions or investigations, where specific antimicrobial treatment is known to improve outcomes, or if certain pathogens are known to portend a worse prognosis (for instance risk of persistence and malnutrition). Therefore future work is needed on several fronts: the creation and refinement of molecular tests for multiple pathogens, including reduction of cost and of laboratory sophistication, and carefully-designed studies to better understand the clinical and epidemiologic implications of molecular results.

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