50 Gastrointestinal Infections

Eileen M. Burd and Benjamin H. Hinrichs

Abstract

Establishing a specific etiology for gastrointestinal infections can be challenging because of the common clinical features and wide variety of causative microorganisms. In many cases, the etiologic agent cannot be determined using traditional diagnostic methods and may result in unnecessary antibiotic use or prolonged periods of illness. Molecular tests provide many advantages over traditional laboratory methods but, with the exception of a few analytes, are still largely in the developmental phase for gastrointestinal pathogens and are not widely used. The main advantages of molecular tests include increased sensitivity and the ability to detect agents which will not grow in culture. To test for all possible gastrointestinal pathogens at one time would require a large panel that would include a variety of bacterial, viral and parasitic agents. Challenges inherent in developing diagnostic molecular panels include ensuring that all variants of a particular microorganism can be detected as well as the rapid evolution of pathogens. In this chapter, the diagnostic merit of molecular tests as well as available tests will be presented for the major groups of gastrointestinal pathogens.

Keywords

Infectious gastroenteritis • Diarrhea • Bacterial gastroenteritis • Viral gastroenteritis • Parasitic gastroenteritis • Laboratory diagnosis • Polymerase chain reaction • Gastrointestinal pathogens panel

Introduction

Gastrointestinal infections are a common global health problem. They most often affect the stomach or intestines and generally result in diarrhea. Most gastrointestinal infections are not serious and resolve without treatment after a few days. In select populations, however, diarrheal diseases carry a high degree of morbidity and mortality. The elderly, young children, and people with chronic illnesses or compromised immune systems can become acutely dehydrated and require medical attention.

Many bacteria, viruses, and parasites can infect the gastrointestinal system. Since symptoms are similar, differentiation among the various etiologies is difficult. The microorganisms that cause gastrointestinal infections vary with the geographic region, degree of economic development, level of sanitation, and hygienic standards. In developed countries like the USA, outbreaks of diarrhea are most often a result of food poisoning. Many common gastrointestinal infections are caused by bacteria, including *Bacillus cereus*, *Campylobacter*, *Salmonella*, and enterotoxigenic *Escherichia coli* that are commonly acquired by eating undercooked foods.

E.M. Burd, Ph.D. (✉)
Pathology and Laboratory Medicine, Microbiology Emory University, Atlanta, GA, USA
e-mail: eburd@emory.edu

B.H. Hinrichs, M.D.
Pathology and Laboratory Medicine, Gastrointestinal Pathology, Emory University, Atlanta, GA, USA

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Highly infectious viruses, such as norovirus, can cause gastroenteritis and account for many foodborne illness outbreaks. Gastrointestinal viruses are relatively stable in the environment and can spread rapidly through person-to-person or fomite contact, particularly in enclosed communities, such as hospitals, dormitories, daycare centers, and cruise ships.

Gastrointestinal parasite infections are typically acquired from ingestion of contaminated food or water. The parasite *Giardia lamblia* is often consumed by hikers who drink untreated stream water. *Cryptosporidium* has been associated with drinking water or recreational water. Outbreaks of *Cyclospora* and *Cystoisospora* (formerly *Isospora*) have been associated with consumption of contaminated food or water. These parasites are more common in tropical and subtropical areas of the world and people traveling to countries where the disease is endemic may be at increased risk for infection.

Identification of the causative agent in clinically significant gastrointestinal infections is important so that appropriate treatment, if any, can be provided. Most gastrointestinal infections will resolve without treatment other than rehydration to replenish lost fluids. Antibiotics are not normally prescribed unless a person is immunocompromised since using any antibiotic or the wrong antibiotic can worsen some infections, prolong the infection, or increase the risk of relapse. Antibiotics may be given for certain bacteria, specifically *Campylobacter*, *Shigella*, and *Vibrio cholerae*, but are not used for uncomplicated cases of *Salmonella* or toxigenic *E. coli*. Gastrointestinal parasitic infestations are treated with appropriate antiparasitic medication to help eliminate the parasite.

Molecular methods can provide rapid and sensitive detection of gastrointestinal pathogens. Molecular amplification is greatly complicated by the presence of a complex and abundant gut microflora and high concentrations of potential PCR inhibitors in diarrheal stool specimens [1]. Approaches to reduce PCR inhibition include dilution of extracted nucleic acids, treatment of samples with chelating agents (Chelex 100, Bio-Rad Laboratories, Hercules, CA), or adding detergents or denaturing chemicals during extraction. Inclusion of amplification facilitators such as bovine serum albumin or betaine also can increase amplification and overcome low levels of contaminants that co-purify with nucleic acids. Due to the high number of possible enteric pathogens with common clinical presentations, multiplexed molecular tests are advantageous. US Food and Drug Administration (FDA)-approved or -cleared molecular tests are available for some gastrointestinal pathogens in single test or multiplex formats.

For some analytes, reference materials are commercially available and can be used for development of molecular tests, limit of detection studies, cross-reactivity studies, positive and negative controls, training, lot-to-lot comparison of reagent test kits, and other purposes. When reference materials are not commercially available, characterized organisms recovered in clinical or research laboratories may be used. Similarly, proficiency testing surveys are available for many gastrointestinal pathogens, including from the College of American Pathologists (Northfield, IL), the Wisconsin State Laboratory of Hygiene (Madison, WI), and the American Proficiency Institute (Traverse City, MI). When proficiency testing surveys are not commercially available, the laboratory director is responsible for arranging at least semi-annual alternative assessment to evaluate the reliability of analytic testing. Appropriate alternative assessment procedures include split sample analysis with a reference or other laboratory, split samples with an established in-house method such as histology, or clinical validation by chart review. In addition to defining the alternative assessment procedures, the laboratory director must set the criteria for successful performance and ensure documentation of all activities.

Bacterial Pathogens

Bacterial Gastroenteritis

Description of Pathogens

Bacterial gastroenteritis is very common and can affect adults and children. Isolated cases of bacterial gastroenteritis can be seen, but illness usually occurs in outbreaks associated with a group of people who ate the same contaminated food. Bacterial diarrhea is particularly common among people from industrialized countries who travel to developing countries. In developing countries, epidemics of bacterial gastroenteritis with significant associated mortality often arise in areas where sanitation and hygienic practices are poor.

Many bacterial pathogens are well-recognized causes of gastroenteritis. In industrialized countries, *Campylobacter*, *Salmonella*, *Shigella*, and Shiga-toxin producing *Escherichia coli* are the leading causes of bacterial gastroenteritis. Other etiologic agents include *Aeromonas*, *Plesiomonas*, *Listeria*, *Clostridium*, *Staphylococcus aureus*, *Yersinia*, *Vibrio*, and others.

Some enteric pathogens produce characteristic symptoms (e.g., "rice water" stools produced by *Vibrio cholerae*) and the clinical features and pathogenic aspects of a gastrointestinal illness can sometimes be suggestive of a specific etiology. Most often, however, the presenting clinical features do not reliably suggest a particular etiology and laboratory tests are needed to identify the specific pathogen.

In general, these bacterial pathogens can be readily cultured from freshly collected stool specimens of infected

patients using a variety of selective and specialized media. Clinical laboratories generally use a limited set of media to recover the most common bacterial pathogens (e.g., *Salmonella*, *Shigella*, and *Campylobacter*) and other culture media are used to identify less common agents on special request (e.g., based on travel history, exposure, etc.). Culture and identification of enteric pathogens is cost-effective but can take several days to provide a diagnosis. Culture has some limitations and it is especially difficult to distinguish nonpathogenic from pathogenic strains of *E. coli*. Recent recommendations suggest that, because of the variety of serotypes and difficulty of distinguishing pathogenic forms from normal flora, laboratories should detect enterotoxigenic *E. coli* using immunoassays that detect the toxin in addition to culture [2].

Clinical Utility of Testing

Bacterial gastroenteritis usually is self-limited, but treatment is required in some cases and improper management can lead to a prolonged course. Identification of an etiologic agent allows for more effective targeted treatment which can reduce overall medical costs, and is useful to differentiate bacterial gastroenteritis from other diseases, such as malabsorption syndromes, inflammatory bowel disease, appendicitis, Crohn's disease, diverticulitis, and other enteropathies, that can present with similar symptoms. Since bacterial gastroenteritis can involve groups of people and a common food source, definitive identification of an etiologic agent can be helpful in prompting epidemiologic investigation and testing of potentially contaminated food by public health laboratories. Current stool culture-based tests for bacterial gastrointestinal pathogens typically require several day turnaround times and may yield poor results, especially if a patient has received antibiotic therapy. Molecular tests, especially multiplexed panels, provide accurate diagnosis of at least the most common causes of bacterial diarrhea from a single specimen in one day.

Available Assays

The ProGastro® SSCS® Assay (Hologic Gen-Probe, San Diego, CA) is an US FDA-cleared multiplex real-time PCR test for five common bacterial gastrointestinal pathogens. The test detects *Salmonella*, *Shigella*, *Campylobacter* (*C. jejuni* and *C. coli* only) nucleic acids and Shiga Toxin 1 (stx1) and Shiga Toxin 2 (stx2) genes. The test includes internal controls and is run on a SmartCycler II (Cepheid, Sunnyvale, CA) real-time PCR instrument with results delivered in 4 h.

The xTAG® Gastrointestinal Pathogen Panel (xTAG® GPP, Luminex Corporation, Austin, TX) is another US FDA-cleared, qualitative, multiplex test that simultaneously detects and identifies some viral and parasitic gastrointestinal pathogens in addition to the major bacterial pathogens in a single sample. The bacterial pathogens and toxins that can be detected using the panel include *Salmonella*, *Shigella*, *Campylobacter*, enterotoxigenic *E. coli*, Shiga-like toxin, and toxigenic *Clostridium difficile*. The assay also detects rotavirus A, norovirus GI/GII, *Giardia*, and *Cryptosporidium*. Results are interpreted as presumptive and must be confirmed by US FDA-cleared tests or other acceptable methods. The CE-marked panel available in Canada and Europe detects *Yersinia enterocolitica*, *Vibrio cholera*, adenovirus 40/41, and *Entamoeba histolytica* in addition to those available in the US FDA-cleared panel.

The BioFire FilmArray™ (bioMerieux, Durham, NC) Gastrointestinal (GI) Panel is US FDA-cleared and detects 23 bacterial, viral, and protozoal pathogens, including some not present on other panels. Analytes on the panel include *Aeromonas*, *Campylobacter*, *Clostridium difficile* (Toxin A/B), *Plesiomonas shigelloides*, *Salmonella*, *Yersinia enterocolitica*, *Vibrio*, *Vibrio cholera*, Enteroaggregative *E. coli*, Enteropathogenic *E. coli*, Enterotoxigenic *E. coli*, Shiga-like toxin-producing *E. coli*, *E. coli* O157, *Shigella*/Enteroinvasive *E. coli*, Adenovirus F 40/41, Astrovirus, Norovirus GI/GII, Rotavirus A, Sapovirus, *Cryptosporidium*, *Cyclospora cayetanensis*, *Entamoeba histolytica*, and *Giardia lamblia* along with internal controls to ensure that all processes have been performed successfully. A stool sample collected in Cary Blair transport medium is inoculated into a reaction pouch that contains all of the reagents necessary for the entire reaction. Separate nucleic acid extraction is not required. The pouch is placed in the FilmArray instrument and nucleic acids are extracted and purified, followed by nested multiplex PCR. The first-stage PCR is a single, highly multiplexed reaction and the second-stage PCR reactions detect the products from the first stage PCR. Endpoint melt curve analysis is used to identify the products that are generated. The instrument tests one sample at a time with hands-on time of approximately 2 min and results available in approximately 1 h.

Diatherix Laboratories, an independent CLIA-certified clinical reference laboratory located in the Hudson-Alpha Institute for Biotechnology in Huntsville, Alabama, offers testing for gastrointestinal pathogens using a proprietary technology called target enriched multiplex polymerase chain reaction (Tem-PCR). The bacterial pathogens included in the panel include *Clostridium difficile*, *Clostridium difficile* toxin B gene, *Campylobacter jejuni*, *Escherichia coli* strain 0157, *Listeria monocytogenes*, *Salmonella enterica*, *Staphylococcus aureus*, *Vibrio cholera*, and *Vibrio parahaemolyticus*.

Molecular tests are expected to play an increasingly important role in the diagnosis of gastrointestinal illnesses. Because of the complexity of gastrointestinal pathogens and

the emergence of variants of these pathogens, future clinical molecular tests will likely include new approaches such as bead-based microarrays, microfluidic systems, and other methods that simultaneously target many more pathogens than current methods allow.

Interpretation of Results

Interpretation of positive results is not generally problematic. Because the asymptomatic carriage rate is extremely low, detection of specific bacterial pathogens in stool in the absence of other enteric pathogens can be considered diagnostic.

Laboratory Issues

Bacterial culture may still be needed for cases where antibiotic susceptibility testing is required. Antimicrobial therapy is indicated for some cases of gastrointestinal illness due to *Salmonella*, *Shigella*, *Aeromonas*, *Yersinia*, and *Vibrio* and some others, but not for *Pseudomonas*, *S. aureus*, or toxigenic *E. coli* [3]. Because of increasing resistance and strain variability, susceptibility testing is recommended to guide therapy.

Reference materials are available from several vendors. Previously characterized positive stool samples or negative samples spiked with well-characterized organisms recovered in the clinical laboratory can be used. Dried genomic nucleic acids are available for some analytes from the American Type Culture Collection (ATCC) (43504D, Manassas, VA) or BEI Resources (Manassas, VA) which is managed by ATCC. The NATtrol™ (ZeptoMetrix Corp, Buffalo, NY) verification set contains all of the analytes in the BioFire GI panel.

Proficiency testing programs that are compatible with molecular methods and accommodate most of the analytes on gastrointestinal pathogen panels are available from several organizations. A combination of programs might be needed to fully accommodate entire testing panels. The College of American Pathologists (Northfield, IL) offers a Gastrointestinal Panel for Molecular Multiplex Testing (GIP) survey that includes *Campylobacter*, *Clostridium difficile* toxin AB, *Cryptosporidium*, Enterotoxigenic *E. coli*, *Giardia*, Norovirus GI/GII, Rotavirus A, *Salmonella*, Shiga-like toxin producing *E. coli* SXT-1 and SXT-2, and *Shigella*. The Wisconsin State Laboratory of Hygiene (Madison, WI) offers a Comprehensive (MC) Bacteriology survey that includes *C. difficile* toxin or antigen as well as enteric pathogen identification. Separate surveys include *C. difficile* (CD), Shiga Toxin (SHG), and Enteric Pathogens (NP) including *Aeromonas*, *Campylobacter*, *E. coli* O157:H7, *Plesiomonas*, *Salmonella*, *Shigella*, *Vibrio*, and *Yersinia*. The American Proficiency Institute (Traverse City, MI) has several programs available that include *Campylobacter*, toxigenic *Clostridium difficile*, Rotavirus, *Giardia*, and *Cryptosporidium* in addition to bacteriology and virology programs.

As molecular tests become more widely used, accommodation will need to be made for reporting of positive results to public health departments. Most state public health departments require notification when certain infectious agents, including many gastrointestinal pathogens, are suspected or identified. This reporting allows public health departments to investigate outbreaks and conduct surveillance studies to assess changes and trends in disease occurrence. For some gastrointestinal bacteria such as *Salmonella* and Shiga-toxin-producing *E. coli*, health departments usually require a culture of the organism to be submitted for definitive identification, typing studies, etc. that help identify common foodborne sources. This policy will need to be modified if molecular testing for bacterial gastrointestinal pathogens becomes widely used in clinical laboratories.

Clostridium difficile

Description of Pathogen

Clostridium difficile is a spore-forming, Gram-positive anaerobic bacillus that is carried by some individuals as a component of the normal intestinal microbiota. Pathogenic strains produce toxin A and/or toxin B, which damage the intestinal mucosa. Toxigenic *C. difficile* is associated with nearly all cases of antibiotic-related colitis and 15–20 % of antibiotic related diarrhea [4, 5].

C. difficile infection (CDI) has become a scourge of hospitals worldwide and is estimated to account for an excess $1 billion to $3.2 billion per year of healthcare costs in the USA [6, 7]. While advanced age and length of hospitalization are directly related to increased risk of CDI, exposure to antibiotics remains the most significant modifiable risk factor [8]. Exposure to antibiotics and subsequent loss of endogenous enteric microbiota is believed to create a favorable environment for the growth of *C. difficile*. While reports of *C. difficile* colonization in both healthy children and adults suggests the possibility of an endogenous source of infection [9, 10], epidemiologic studies have established the significance of the organism as a transmissible nosocomial pathogen [8, 11].

Disease is caused by the production of toxins A and B, which are encoded by the genes *tcdA* and *tcdB*, respectively. Not all strains carry these genes, and demonstration of the ability to produce toxin is an essential criterion for the diagnosis of CDI. Recent studies highlight the importance of toxin B over toxin A in disease pathogenesis [12], and most clinical assays focus on the detection of *tcdB* gene sequences. Two regulatory genes, *tcdC* and *tcdD*, are hypothesized to negatively influence the expression of *tcdA* and *tcdB*, and together with the toxin genes are part of the chromosomally encoded region known as the pathogenicity locus (PaLoc) [8].

Since 2001, several US and Canadian hospitals reported outbreaks of CDI associated with increased disease severity [13, 14]. Epidemiologic studies revealed a high percentage

of cases were caused by a strain referred to as BI/NAP1/027, named in reference to typing results for restriction endonuclease analysis (REA), pulsed field gel electrophoresis (PFGE), and PCR-ribotyping, respectively. The BI/NAP1/027 strain carries 18-bp and 1-bp (nt 117) deletions within the *tcdC* gene, and these deletions are speculated to result in the formation of an abnormal *tcdC* protein with a loss of regulatory function [13]. The resultant loss of negative regulation may lead to an increase in toxin formation and greater virulence [13]. Supporting this theory is the observation that isolates of the BI/NAP1/027 strain produce increased amounts of toxin A and B in vitro [15]. An additional toxin known as the binary toxin CDT is present in the BI/NAP1/027 strain as well as 6 % of *C. difficile* isolates, and is encoded by two chromosomal genes, *cdtA* and *cdtB*, located outside of the PaLoc [13]. Although production of the binary toxin is associated with the more virulent BI/NAP1/027 strain, its role in pathogenesis is not well established [8, 16].

Clinical Utility of Testing

Rapid and accurate diagnosis of CDI is critical not only for the timely treatment of individual patients, but also for preventing the spread of nosocomial disease. The diversity of tests available makes possible a number of diagnostic algorithms. None of these testing strategies has performed optimally to date, leading a number of investigators to suggest the adoption of the highly sensitive and specific PCR-based assays. Several studies have evaluated the effects of implementing nucleic acid testing. Algorithms examined include PCR assays as confirmatory tests of glutamate dehydrogenase (GDH)-positive samples, as reflex tests for GDH-positive, toxin-enzyme immunoassays (EIA) negative samples, and as stand-alone assays for direct testing of stool samples. A comparison of these testing algorithms to those using toxin-EIA only, GDH followed by toxin-EIA, and GDH/toxin-EIA followed by cell culture cytotoxin neutralization (CCCN) testing of toxin-EIA negative samples indicates a clear trade-off between sensitivity and cost [17]. The cost per test for all strategies using PCR-based assays was significantly greater than the most expensive non-PCR-based testing algorithm ($35.22 vs $24.41, respectively) [17]. However, strategies using PCR as confirmation of GDH-positive samples or as a reflex test for GDH-positive, toxin-EIA negative samples detected an additional 89 toxigenic *C. difficile* samples over a 1 year period that were missed by algorithms using traditional testing methods [17]. Furthermore, stand-alone, direct PCR testing of stool samples detected an additional 138 positive specimens missed by even the most sensitive non-PCR-based testing algorithm. Importantly, all diagnostic strategies using PCR provided results for the majority of samples (>83.7 %) in less than 1 h and in less than 5 h for the remainder [17]. These rapid turnaround times are in contrast to algorithms relying on CCCN as a reflex test for GDH-positive, toxin-EIA negative samples, which required as long as 48 h for 12.3 % of samples tested [17].

An optimal testing strategy balances the number of CDI cases detected with total costs and turnaround time. Although the additional costs of algorithms employing PCR are significant, use of these assays would likely allow for the earlier detection of disease. In turn, earlier detection of disease could prevent the spread of nosocomial infection and decrease the total number of CDI cases. In addition, rapid detection allows for the timely institution of treatment and possibly shortened hospital stays. The assessment of total cost, therefore, must consider both expenses related directly to testing in addition to costs savings realized as a result of lowering the incidence of nosocomial disease and decreasing time of hospitalization.

In an effort to determine how different diagnostic algorithms might affect isolation practices of patients with suspected CDI, Tenover et al. applied the findings of several studies to a theoretical model of 1,000 patients with 10 % disease prevalence [16]. The results confirm the poor performance of strategies relying solely on GDH/toxin-EIA testing as the number of patients placed in isolation with true CDI nearly matches the numbers of patients without the disease [16]. Furthermore, 45 patients with CDI are not identified, and therefore, are not placed into proper infection control isolation [16]. Algorithms that reflex to toxigenic culture or CCCN after GDH/toxin-EIA testing detect more cases of CDI, but still produce high numbers of false-positive results (55 patients). Using toxigenic culture as a reflex test produced values for sensitivity and specificity statistically equivalent to PCR-based reflex testing; however, the length of time required to produce final results would likely lead to excessive costs. Using PCR-based assays as stand-alone tests detected the greatest number of CDI cases (95 %), and led to the unnecessary isolation of only 36 patients [16]. While these results further confirm the superior diagnostic performance of nucleic acid testing either as a reflex test for GDH-positive, toxin-EIA negative samples or as a stand-alone method, the authors of the study recommend additional investigations examining the cost-effectiveness of these strategies [16].

A potential concern for diagnostic algorithms using GDH as a screening test is highlighted by a report from Larson et al who identified four (1.9 %) of 211 GDH-negative samples with the *tcdB* gene by direct PCR testing [17]. These four apparent false-negative samples also were negative by CCCN, but confirmed as containing toxigenic *C. difficile* by toxigenic culture. The results are consistent with studies demonstrating lower sensitivities and negative predictive values for an algorithm combining GDH and PCR compared to utilizing just PCR [18].

While not contributing directly to patient care, typing methods have provided important insights into CDI epidemiology. In addition to aiding determinations of infection source, reservoir, and mode of transmission, typing methods allow investigators to correlate abnormally severe clinical behavior with putative virulence factors. Both phenotypic and genotypic methods are used; however, phenotypic methods are generally less reproducible and some strains have not been able to be phenotyped [19].

Available Assays

The optimal means of diagnosing CDI is still evolving, and current recommendations put forth by several professional organizations are conflicting [8, 20]. In the past, CCCN was regarded as the gold standard because of the ability to directly identify the presence of toxin B. However, the inability of CCCN to detect a large number of CDI cases is well documented, and the clinical utility is further diminished by a lengthy turnaround time (24–48 h) [17, 21]. Bacterial culture followed by a sensitive and specific toxin assay performed on the isolated organism (toxigenic culture) has replaced CCCN as a reference method in many studies [8, 20, 22]. Toxigenic culture has demonstrated superior sensitivity compared to CCCN, but because toxin production is assessed only after the organism has been grown in vitro, the clinical meaning is not clear [20]. Unfortunately, similar to CCCN, toxigenic culture requires considerable technical expertise, and has an average turnaround time of 3–7 days [23].

EIA for the detection of toxins A and B (toxin-EIA) are rapid and easy to use alternatives to culture-based testing, and are currently the most commonly used tests in the USA [8, 21]. However, recent studies comparing their performance to toxigenic culture indicate sensitivities ranging from 32–67 % [24, 25], thus preventing their use as a reliable screening or stand-alone test [8, 20]. EIA tests are available for the detection of GDH, a constitutively expressed enzyme produced by nearly all *C. difficile* strains as well as some non-*C. difficile Clostridium* sp. [16, 26]. These GDH EIAs demonstrate sensitivities of greater than 90 %, and diagnostic algorithms often use these assays as initial screening tests [26]. However, GDH EIAs detect both toxigenic and non-toxigenic strains, and GDH-positive samples must be confirmed with an assay demonstrating toxin production. Options for confirmation include toxigenic culture, CCCN, and toxin-EIAs. However, due to the low sensitivities of CCCN and toxin-EIAs, a number of cases would likely be missed.

Molecular tests have emerged as additional options for both confirmatory and stand-alone testing. These methods generally demonstrate excellent sensitivities and specificities, and most assays are capable of delivering results in 1–3 h [16]. Several tests are US FDA-cleared, and additional assays will likely become commercially available in the near future. At this time all US FDA-cleared assays are qualitative, although quantitative testing is technologically possible with real-time PCR. Most US FDA-cleared assays are based on real-time PCR. However, other novel methods such as helicase-dependent amplification (HDA) and loop-mediated isothermal amplification (LAMP) also are employed. In addition, the majority of commercially available real-time PCR assays target solely the *tcdB* gene. US FDA-cleared multiplex PCR assays target *tcdB* as well as variable *tcdA*, *tcdC*, and *cdt* gene sequences and the single base pair deletion at nucleotide 117 in the *tcdC* gene associated with the 027/NAP1/B1 strain. Initial studies evaluating these US FDA-cleared assays are promising; however, the 2010 Update by the Society for Healthcare Epidemiology of America and the Infectious Diseases Society of America stated the need for further studies before recommending molecular assays for routine testing [8]. Lastly, CDI screening also may be performed using the US FDA-cleared xTAG® Gastrointestinal Pathogen Panel (Luminex Corporation), a multiplex test that simultaneously detects 11 gastrointestinal pathogens including *C. difficile* and other major bacterial, viral, and parasitic pathogens. Published reports evaluating the US FDA-cleared version of this assay are currently lacking.

As stated above, most real-time PCR assays target *tcdB*; however, interest in detecting the hypervirulent BI/NAP1/027 strain has spurred the development of assays that evaluate *tcdC* [12]. Some investigators contend that *tcdC* may be used as a surrogate target for *tcdA* and *tcdB*, and excellent correlation between the presence of *tcdC* and *tcdA* and/or *tcdB* has been demonstrated [25]. The authors also report the ability to detect deletions in *tcdC*, including the 18-bp deletion associated with the BI/NAP1/027 strain. Nevertheless, the assay was unable to discriminate the 18-base pair (bp) deletion from a 39-bp *tcdC* deletion not currently associated with an epidemic strain, and therefore, cannot be used to reliably detect the presence of the BI/NAP1/027 strain [25].

The US FDA has cleared assays using non-PCR amplification methods such as LAMP and HDA. LAMP assays amplify target DNA isothermally, and identify successful amplification by detecting an increase in turbidity due to the build-up of a reaction by-product. HDA assays also amplify isothermally and use helicase enzymes to separate DNA strands rather than thermal denaturation [27]. These assays are attractive to laboratories that are not able to purchase expensive thermal cyclers or detection systems. Like real-time PCR methods, LAMP- and HDA-based assays are rapid and demonstrate excellent sensitivities and specificities [27–29]. The *illumi*gene *C. difficile* assay is US FDA-cleared for testing of symptomatic children ages 1–2 years. Other US FDA-cleared nucleic acid detection assays have not received clearance for this age group. As asymptomatic colonization

of children under the age of two is well documented [9], testing of this age group is controversial.

Finally, genetic typing methods for epidemiologic analysis may be broadly categorized into methods using REA, PCR, or direct sequencing [19]. These methods require DNA extracted from a single clone, and therefore, culture should be obtained when there is concern either of an outbreak or of a particularly virulent toxigenic strain [19]. Genetic typing techniques have the ability to discriminate and characterize a broad range of epidemic and non-epidemic *C. difficile* strains; however, currently there is focused interest in the epidemiology of the BI/NAP1/027 strain. Two multiplex PCR assays that detect the single bp deletion at nucleotide 117 in the *tcdC* gene associated with this hypervirulent strain have recently been US FDA-cleared. In both cases, detection of the BI/NAP1/027 strain is US FDA-cleared for epidemiologic investigations only.

Interpretation

A positive real-time PCR test is generally diagnostic of toxigenic *C. difficile* in a patient displaying typical signs and symptoms of CDI. While it is possible that a patient may be colonized with toxigenic *C. difficile* and suffer diarrhea caused by a different etiology, this situation is likely to be rare [16].

PCR may detect toxigenic *C. difficile* even though non-toxigenic *C. difficile* or negative growth is reported by culture. Many of these cases are positive by GDH EIA, toxin-EIA, or CCCN, and thus are regarded as true positives. Culture may fail to detect growth because of significant time delays between sample collection and testing, concurrent antibiotic treatment at time of collection, or laboratory issues as discussed below [18].

Positive predictive values for real-time PCR assays may be as low as 84 %, and demonstrate that not all positive results are indicative of CDI [18]. Studies reporting the occurrence of isolated PCR-positive results also suggest PCR-based assays may be too sensitive. Additional studies correlating the clinical outcomes of patients who test negative by conventional methods but positive by PCR are needed to improve diagnostic accuracy.

The occurrence of a positive PCR result in an asymptomatic patient indicates colonization with toxigenic *C. difficile* and is well documented [10]. For this reason the testing of asymptomatic patients, except for epidemiologic purposes, is not recommended [9].

Negative PCR results generally indicate the absence of CDI, as evidenced by high negative predictive values for these assays. Recommendations made by the European Society of Clinical Microbiology and Infectious Diseases state that a single negative result from a *tcdB* PCR assay, a GDH EIA, or toxin-EIA may be used to rule out the presence of toxigenic *C. difficile* [20]. While negative PCR results may occur in samples testing positive by conventional detection methods, this is an uncommon occurrence and may be due to one of the reasons discussed below.

Laboratory Issues

The sensitivity of real-time PCR assays may be as low as 77.3 % and negative results are speculated to occur for a number of reasons [22]. Samples negative by PCR, but positive for toxigenic isolates may contain substances inhibitory to PCR amplification. The detection of PCR inhibitors is aided by the incorporation of internal controls into all US FDA-cleared real-time PCR assays. Other investigators propose that negative PCR tests may be due to sampling error of stool, a known heterogeneous testing medium, when low numbers of organisms are present [18, 30].

A concern regarding the clinical significance of PCR stems from the fact that PCR merely detects the presence of a gene and does not evaluate gene expression. While it is theoretically possible for PCR to detect *C. difficile* carrying *tcdB* that is not expressed, testing only unformed stool specimens from patients with a clinical suspicion of CDI may help avoid this issue [16]. So far, no published studies have evaluated this point directly.

Real-time PCR assays targeting *tcdB* are believed to comprehensively detect all current strains of toxigenic *C. difficile* as *tcdB* negative strains or strains with significant deletions in *tcdB* do not exist naturally [12, 31]. Strain-to-strain *tcdB* sequence variability resulting in poor primer binding is occasionally cited as a concern for the ability of toxin B PCR assays to sensitively detect the wide range of *C. difficile* strains. While significant sequence variation within the *tcdB* gene is reported [31], most currently targeted *tcdB* sequences appear conserved across the range of strain types [16]. Therefore, the effect of *tcdB* sequence variation on sensitivity of toxin B PCR assays is expected to be minimal [16].

Also of concern is the potential evolution of a novel toxigenic *C. difficile* strain with an altered *tcdB* sequence as a result of genetic drift. Although most toxigenic *C. difficile* strains contain an intact *tcdB* gene, the detection of a strain deficient in at least a portion of the *tcdB* gene is reported in a case of recurrent CDI [32, 33]. While the report of a clinically significant *tcdB* deficient strain reinforces the need to be vigilant for the development of *tcdB*-negative, *tcdA-positive* strains affecting the clinical performance of toxin B PCR assays, the occurrence of such variant strains is currently rare [22, 33]. Multiplex real-time PCR assays, with the ability to simultaneously detect several different sequence targets (e.g., *tcdA* and *tcdB*, *cdtA*, *cdtB*, and *tcdC*) may decrease the likelihood of detection failure due to primer sequence mismatches [23].

The recognition of clinically significant toxin A negative, toxin B positive strains also is cited as a concern for the ability of LAMP assays to comprehensively detect all CDI cases.

Although four toxin A negative, toxin B positive strains are currently recognized, only one strain, toxinotype VIII, has been associated with significant numbers of CDI cases [34]. The *tcdA* gene of toxinotype VIII contains both a 1.8 kb deletion in addition to a nonsense mutation resulting in a truncated toxin A protein [31, 34]. Despite the modified toxin A gene sequence of toxinotype VIII, a recent study found that three strains of toxinotype VIII tested on the *illumi*gene *C. difficile* assay were detected [35]. Several studies report samples that are PCR-positive, but negative by toxigenic culture.

Although toxigenic culture is a more sensitive reference method than CCCN, instances of detection failure are documented [18]. Reasons for detection failure may be clinical as mentioned above, but also may involve factors related to laboratory handling of specimens. Results of toxigenic culture for *C. difficile* may be adversely affected by long delays between collection and testing of specimens, the failure to enrich for spores, or the loss of spore viability during the spore enrichment process affecting only particular strains [18, 22]. In addition, culture is speculated to occasionally fail to detect toxigenic strains as a result of overgrowth by a non-toxigenic strain, as studies have reported the presence of multiple strain types in patient samples [22, 36].

A *C. difficile* verification panel that includes ribotype 027 and *C. sordellii* as a negative control is commercially available (ZeptoMetrix Corp, Buffalo, NY). A panel of 8 *C. difficile* strains, each with a different toxinotype as well as freeze-dried, well-characterized *C. difficile* strains and genomic DNA from those strains are available from ATCC (Manassas, VA). The ACCURUN 501 *C. difficile* Control (SeraCare Life Sciences, Gaithersburg, MD) contains inactivated organisms in a human synthetic stool matrix. The control set contains *C. difficile* NAP1/027/B1 hypervirulent strain, two toxigenic *C. difficile* strains, and *C. sordelli* as a negative control. Proficiency testing samples for *C. difficile* molecular tests are available from the College of American Pathologists, the Wisconsin State Laboratory of Hygiene, and the American Proficiency Institute.

Tropheryma whipplei

Description of Pathogen

The etiological agent of Whipple's disease is *Tropheryma whipplei*, a bacterium present in the environment, sewage, human stool, and saliva, but whose entire ecological distribution is yet to be characterized [37, 38]. In accordance with these findings, a fecal-oral route of transmission has been proposed [38]. Evidence also exists for the ability of the organisms to asymptomatically colonize the upper gastrointestinal tract, as PCR has identified *T. whipplei* DNA in the saliva and gastric juice of 35 % and 11.4 % of individuals, respectively, without evidence of Whipple's disease [39, 40].

Whipple's disease, however, is rare, and only an estimated 1,000 cases have been described [38]. Its pathogenesis is poorly understood, and while genetic risk factors have been proposed, none have been confirmed [38]. The most common presentation of disease includes symptoms related to malabsorption such as diarrhea and weight loss, although a long history of nonspecific complaints, often including arthralgias, is typical [38, 40]. These vague and chronic symptoms may last an average of 6 years before clinical signs more characteristic of the disease appear [38]. Atypical cases lacking classic gastrointestinal symptoms and involving the cardiovascular system and the central nervous system (CNS) as well as other organ sites may occur in up to 15 % of those affected [38]. The long time period before typical symptoms are manifested in addition to the high percentage of unusual presentations often results in a delay of treatment. Early diagnosis and the initiation of antibiotics are critical to avoiding long-term morbidity, and, therefore, improved detection methods are needed.

Clinical Utility of Testing

The diagnosis of Whipple's disease is made primarily by histological examination of tissue biopsies; however, since the 1990s, PCR has played an increasing role in diagnosis. Culture and serological methods have recently been developed as diagnostic tools; however, their availability is limited [37, 38]. In the past, electron microscopy (EM) was commonly used to demonstrate the characteristic trilaminar bacterial cell wall; however, its utilization is declining [41].

The optimal use of PCR in establishing a diagnosis of Whipple's disease is debated. Some reports recommend PCR testing in parallel with the procurement of biopsies, citing the lack of optimal specificity of histology and PCR when used alone [38]. Others advocate the use of PCR only when biopsies fail to indicate disease, although this is not supported by the low rate of PCR positivity in histologically negative duodenal biopsies [42].

Intestinal biopsies from patients without gastrointestinal symptoms may be negative by PCR, and thus clinical symptoms in atypical cases should guide the selection of samples for PCR analysis [42]. In addition to duodenal biopsies, PCR testing has proven useful when performed on a number of different specimen types including lymph nodes, cardiac valves, synovial fluid, cerebrospinal fluid (CSF), and vitreous humor [37]. CSF has tested positive by PCR methods in several patients without CNS symptoms, and may indicate the need for antibiotics with good CNS penetration [41]. While saliva, feces, and blood may be positive in patients with Whipple's disease, higher rates of background positivity makes the utility of testing these specimens uncertain [37, 43, 44].

No biological marker or test is currently available to determine the required duration of treatment for Whipple's disease [38]. Nevertheless, monitoring response to treatment using PCR appears to have utility for predicting outcome [41, 44]. PCR positivity after treatment correlated with a higher likelihood of relapse; however, the positive predictive value was only 58 % [45]. Likewise, while negative post-treatment PCR results have been associated with remission, a significant number of patients without detectable *T. whipplei* DNA in intestinal biopsies have developed recurrent disease [46].

Molecular methods for genetic subtyping of *T. whipplei* are limited to the research setting, as currently no correlations are established between subtype and geographic location or specific clinical manifestations [44].

Available Assays

Duodenal biopsies demonstrating expansion of the lamina propria by macrophages filled with Periodic acid-Schiff (PAS)-positive bacterial fragments is the classic histological finding of Whipple's disease, and is observed in the majority of Whipple's disease patients [40]. While relatively specific when identified in a patient with typical Whipple's symptomatology, the protean symptoms of this pathogen necessitate the consideration of other infectious diseases, such as *Mycobacterium avium* complex and *Rhodococcus equi*, as well as noninfectious disorders, all of which may have overlapping histological appearances [41, 44]. Biopsies obtained from other anatomic sites demonstrating macrophages filled with PAS-positive material are even less specific, and must be interpreted with caution [37, 40, 44]. Immunohistochemistry for *T. whipplei* performed on paraffin-embedded tissues has recently been developed and has greatly improved both the sensitivity and specificity of histological diagnosis [38]. Lastly, intestinal biopsies may be non-contributory due to the patchy nature of disease and the possibility for diagnostic material to be confined deep within the submucosa and not usually evaluated in superficial mucosal biopsies [41, 44].

The existence of disorders with overlapping histological findings and the possibility for biopsies to miss disease supports the diagnostic role of PCR-based methods. While reports of sensitivity are limited, several studies document PCR positivity in nearly all cases of histologically proven disease [42, 45]. Detection of *T. whipplei* DNA from negative intestinal biopsies by PCR-based tests highlights the diagnostic sensitivity and utility of molecular methods [42, 44].

Molecular detection of *T. whipplei* is primarily using PCR methods including conventional [45], nested [39], semi-nested [47], and real-time PCR [48–50]. Primers targeting 16S rDNA, 16S-23S rDNA intergenic spacer, 23S rDNA, and *rpo*B sequences are commonly used [44]. Assay sensitivity and specificity varies according to the amplification target and the PCR method.

Conventional PCR assays provide qualitative results, and detection techniques are time consuming, requiring 2 or more days to perform. Ethidium bromide-stained gel electrophoresis may be used to detect amplified bands of characteristic size; however, an additional confirmatory identification step is recommended [38]. Options include Southern hybridization using sequence-specific fluorescent oligonucleotide probes or direct sequencing techniques [38]. Nevertheless, these assays can perform adequately, and a study evaluating a conventional PCR assay using paraffin-embedded tissue from patients with histologically confirmed Whipple's disease demonstrated a sensitivity and specificity of 96.6 % and 100 % respectively [45].

Semi-nested or nested PCR methods generally allow for a lower limit of detection than conventional PCR assays; however, these methods are associated with a higher risk of contamination due to the required handling of amplification products [44]. An additional disadvantage of these methods includes their longer turnaround time compared to real-time PCR methods.

Real-time PCR methods are more rapid and less prone to contamination than conventional, semi-nested, and nested PCR assays. In addition, real-time PCR assays provide quantitative results, which help differentiate true infection from contamination or low-level colonization [49]. A study comparing the performance of a LightCycler® (Roche Molecular Systems, Branchburg, NJ) real-time PCR assay to a conventional PCR assay demonstrated good correlation of results; however, the turnaround time was significantly shorter for the real-time PCR assay (3.5 h vs 2–3 days) [48].

No tests are FDA-cleared or -approved for the detection of *T. whipplei*; however, amplification of *T. whipplei* DNA by PCR in blood, CSF and tissues is available from some reference laboratories.

Interpretation of Results

A positive PCR result in the setting of classic Whipple's disease symptoms and biopsy findings is generally confirmatory. Correlating positive PCR results with clinical findings is especially important in atypical presentations and when histology is non-contributory. The need for clinical correlation is highlighted by occasional studies identifying the presence of organisms in asymptomatic adults. Most of these studies produced results using nested and semi-nested PCR assays [39, 43], which are associated with a high contamination risk [44]. Such unexpected positive results could be due to environmental contamination, asymptomatic colonization, or nonspecific amplification of non-*T. whipplei* DNA. These findings have not been confirmed as several other studies have found that PCR performed on intestinal biopsies is consistently negative in patients undergoing endoscopy to investigate conditions other than Whipple's disease [42, 49]. The ability for real-time PCR to produce

quantitative results may allow differentiation of true infection from contamination or asymptomatic colonization [49]; however, specific ranges have not been determined.

Negative PCR results in patients diagnosed with Whipple's disease histologically may be due to DNA degradation as a result of the DNA extraction process, the presence of PCR inhibitors, or damaged DNA from formalin-fixation [44]. In addition, negative PCR results may prompt consideration of other infectious etiologies capable of producing histological findings similar to Whipple's disease [44].

Laboratory Issues

As mentioned above, DNA degradation during extraction from tissues and other clinical samples may cause false-negative results. Choosing and validating an appropriate extraction method, therefore, is critical, and commercial extraction kits include chaotropic lysis (Isoquick Kit, Orca Research, Bothell, WA, USA), Pure-Gene protocol (PureGene Kit, Flowgen Instruments Ltd., Lichfield, UK), and QIAamp DNA binding columns (QIAGEN, Hilden, Germany) [44]. Extraction efficiency may be evaluated using primers targeting human gene sequences as internal controls.

PCR inhibitors may cause false-negative results. Similar to the evaluation of extraction efficiency, the presence of PCR inhibitors may be identified using primers targeting ubiquitous human genes as internal controls [44].

PCR testing can be performed retrospectively on paraffin-embedded tissue biopsies; however, the use of fresh or frozen specimens provides more sensitive results [42].

Suspensions of organism are often used as reference material but are not currently commercially available. Proficiency testing surveys also are not commercially available.

Helicobacter pylori

Description of Pathogen

Helicobacter pylori is a spiral-shaped and flagellated, Gram-negative bacterium that can be found in the stomach of some individuals. The *H. pylori* genome is approximately 1.64–1.67 million bp with 1,515–1,590 predicted protein-coding sequences. *H. pylori* has unusually high levels of genetic variation between strains due to a natural DNA uptake system that can incorporate very large fragments of exogenous DNA into the *H. pylori* genome.

H. pylori infection is a leading cause of gastric and duodenal ulcers, and is strongly associated with gastric malignancies such as gastric adenocarcinoma and gastric mucosa-associated lymphoid tissue (MALT) lymphoma [51]. While the clinical course of infection may be variable and depends on both host and microbial factors, the organism most often produces a chronic infection manifested as chronic gastritis [51, 52]. Treatment aims to completely eliminate the organism, and eradication cures the majority of both duodenal and gastric ulcers. Although treatment results in regression of most low-grade gastric MALT lymphomas [53, 54], treatment effectiveness in reducing the risk of gastric adenocarcinoma is less clear [51, 53].

H. pylori infection rates are greatest in developing countries due to lower socioeconomic conditions; however, prevalence estimates in the USA are high, reaching 30–40 % [55]. Strains demonstrating antibiotic resistance limit the effectiveness of standard eradication regimens, which usually include clarithromycin, either amoxicillin or metronidazole, and a proton-pump inhibitor (PPI) [51, 56]. Prevalence of clarithromycin resistance is estimated to be 10–15 % in the USA, and resistance results in a 70 % reduction in the eradication rate [56]. While resistance to metronidazole occurs more frequently (20–40 %), eradication rates of resistant strains are decreased by only 25 % [56]. Strains resistant to amoxicillin and second-line antibiotics such as tetracycline are much less common, but their presence could alter the effectiveness of rescue therapies [56].

The high prevalence of *H. pylori* infection and antibiotic resistance, in addition to the clear benefit of eradication therapy necessitates the use of reliable diagnostic tests. Molecular methods consisting primarily of PCR-based assays are both rapid and sensitive, and while useful for the general diagnosis of *H. pylori* infection may also play an important role in special clinical situations such as acute bleeding. Furthermore, although antibiotic resistance may be detected effectively using conventional phenotypic testing of *H. pylori* cultures, genotypic susceptibility testing of cultures and biopsy specimens offers a fast and reliable alternative.

Clinical Utility of Testing

Diagnostic testing for *H. pylori* is indicated in patients with either active or previously documented peptic ulcer disease, low-grade gastric MALT lymphoma, and in certain cases of dyspepsia not yet investigated by endoscopy [57]. The diagnosis of *H. pylori* infection may be established using a wide variety of diagnostic tests and the choice of test is largely determined by clinical factors such as whether or not the patient requires upper gastrointestinal endoscopy [57].

Tests utilized with endoscopy and regarded as invasive include culture, histology, rapid urease tests (RUTs), and PCR assays performed on biopsy material. Noninvasive tests not requiring endoscopy include serology, the urea-breath test (UBT), and stool tests such as antigen assays and PCR. Each test has particular disadvantages and comparing diagnostic performance is made difficult by the fact that no single test method is regarded as the gold standard. As a substitute for a gold standard, studies comparing different assays often designate true positives as those samples yielding positive results with two or more testing methods.

While certain non-molecular methods such as the UBT, histology, and RUTs offer sufficient sensitivity for routine testing, PCR-based molecular methods performed on tissue biopsies offer even greater sensitivity and may identify true positives missed by RUT and histology [58–62]. This increased sensitivity has the greatest utility in evaluating patients with bleeding peptic ulcers where non-molecular assays perform poorly [58, 63, 64]. Real-time PCR assays may also improve patient care by offering quantitative results. High bacterial densities are associated with lower eradication rates, and, therefore, quantitative measurements by real-time PCR may be used to identify patients who could benefit from a modified, more intense treatment regimen [65]. In addition, PCR-based assays for the detection of *H. pylori* DNA in fecal specimens represent potentially simple and noninvasive methods for establishing the existence of infection and for proving successful eradication. However, interpreting results of these tests requires the clinician to be aware of complicating factors that are specific to assays performed on stool. These issues are discussed in detail below.

While culture is currently the gold standard for antibiotic susceptibility testing, the sensitivity of these methods may be reduced due to the fastidious nature of *H. pylori*, overgrowth of cultures by bacterial contaminants, and by low numbers of viable *H. pylori* organisms in the post-treatment period [66]. In addition, cultures may take several days for growth. Fortunately, effective and rapid genotypic susceptibility testing methods are available and may be applied to both culture and biopsy specimens.

Numerous studies have looked for an association between the presence of virulence or pathogenic factors and severity of disease. The *cagA* and *vacA* genes are most commonly implicated, and certain alleles of these genes are associated with more severe gastritis as well as higher rates of peptic or duodenal ulcer disease and gastric adenocarcinoma [67–69]. While these pathogenic factors can be detected by molecular methods, including conventional and multiplex PCR, the clinical utility of testing is questionable [70–72]. Confounding variation in host genetic and environmental factors, in addition to discrepant results when comparing different geographic regions and ethnicities, undermines a clear relationship between the presence of pathogenic factors and disease severity [73, 74]. The lack of a clear association has prevented development of clinical guidelines recommending testing of these pathogenic factors for patient care [75].

Available Assays

Several laboratory developed tests (LDTs) for the detection of *H. pylori* DNA in tissue biopsies and stool have been described and include amplification methods such as conventional, nested, multiplex, and real-time PCR, as well as FISH. No US FDA-cleared tests are available. Amplification targets include genes related to the production of virulence factors such as urease (*ureA*), phosphoglucomutase (*glmM*, formerly named urease C (*ureC*)), and *vacA*, as well as species-specific sequences of 16S rRNA and 23S rRNA. Other genes targeted include the sequences encoding the 26-kDa species-specific protein antigen (SSA) and heat shock protein (HSP60), as well as random *H. pylori* genome sequences. Sensitivity and specificity of the assays are determined largely by target gene and primer choice, but also depend on the type of PCR method. Conventional PCR assays provide qualitative results and perform the same or better than conventional detection methods [58]. Detection of PCR amplicons is usually achieved with ethidium bromide-stained gel electrophoresis. Such detection methods require the open handling of PCR products, and, therefore, have a greater risk of contamination than real-time PCR methods.

Real-time PCR methods provide quantitative results, are generally more rapid and sensitive than conventional PCR assays, and also involve less contamination risk [60]. Assays using a hemi-nested or nested design generally allow for a lower limit of detection than conventional PCR assays [76, 77], and achieve similar levels of detection when compared to quantitative RT-PCR (RT-qPCR) assays [61]. Disadvantages of nested and semi-nested designs compared with RT-qPCR include more technologist time and a higher risk of contamination due to handling of amplification products [61].

Multiplex PCR methods combining several different primer targets demonstrate greater sensitivity than conventional testing methods [59]. In addition, a multiplex assay for the detection of both *H. pylori* and *Helicobacter heilmannii-like* organisms may be useful for determining prevalence of disease due to the latter, less common organism [78].

PCR-based assays for the detection of *H. pylori* DNA in fecal specimens frequently use in-house developed capture-probe systems or QIAamp DNA extraction stool kits (Qiagen, Germantown, MD) to obtain purified DNA and to reduce PCR inhibitors [56]. Capture-probe techniques use biotinylated oligonucleotide probes targeting the *H. pylori* 16S rRNA gene [56]. After overnight incubation, the desired gene fragment is harvested using paramagnetic polystyrene beads coated with streptavidin [56, 79]. Gene-capture methods used with conventional PCR targeting 16S rRNA *H. pylori*-specific sequences have demonstrated sensitivities ranging from 75 to 100 % [79, 80]. The QIAamp DNA stool kit has been shown to perform well when used in a semi-nested PCR assay targeting 23S rRNA gene sequences, but results have varied when detecting other genes [56, 81]. Lastly, a filtration-based extraction technique used by Russo et al. demonstrated excellent sensitivity (95.6 %) and specificity (100 %) using a conventional PCR assay with *ureA* primers [82].

Most antibiotic resistance among *H. pylori* strains is due to chromosomal mutations, and therefore, amenable to detection

Table 50.1 *H. pylori* antibiotic resistance mutations detectable by described assays

Antibiotic	Resistance gene	Nucleotide change	Amino acid change
Clarithromycin	23S rRNA	A2142G	NA
		A2143G	NA
Ciprofloxacin	gyrA	C(T) to A	N87K
		C(T) to G	N87K
		A to G	D91G
		G to T	D91Y
		G to A	D91N
Tetracycline	16S rRNA	A926G	NA
		A926C	NA
		A926T	NA
		A928C	NA

NA not applicable

by molecular means (see Table 50.1). *H. pylori* resistance to clarithromycin and other macrolides is caused by point mutations at two nucleotide sites (A2142G and A2143G) within the 23S rRNA gene resulting in decreased ribosomal binding of the antibiotic [52]. Clarithromycin resistance mutations may be detected using several different molecular methods such as restriction fragment length polymorphism (RFLP), pyrosequencing, fluorescence in situ hybridization (FISH), and real-time PCR [56]. PCR followed by RFLP and real-time PCR methods are used most often [56].

RFLP-based assays for the detection of clarithromycin resistance take advantage of the fact that antibiotic resistance mutations create restriction sites within the 23S rRNA gene not present in susceptible strains. Conventional PCR using 23S rRNA specific primers produces amplicons, which when cleaved with restriction endonucleases and visualized by agarose gel electrophoresis create a pattern composed of two bands instead of one. While conceptually simple, these methods are more time consuming than real-time PCR assays.

Real-time PCR methods can detect clarithromycin resistance mutations directly from biopsy specimens with excellent sensitivity and rapid turnaround times of 1–4 h [66, 83–85]. An assay described by Gibson et al. uses fluorescent-labeled probes complementary to the clarithromycin-sensitive 23S rRNA gene sequence [86]. Resistance mutations result in mismatched bases between the probe and target, and melting curve analysis reveals a lower peak melting temperature for the mismatched hybrid than a fully complementary probe and target hybrid [86]. This assay has good concordance with culture-based methods [66, 84], but also identified susceptibilities for an additional 28 patients whose cultures were negative. Of the 28 additional susceptibility results rendered, 21 had resistance genotypes. Another assay design using a biprobe system was tested on 200 patients who failed eradication therapy. The assay detected resistance genotypes with a sensitivity and specificity of 98.4 % and 94.1 %, respectively, when compared to culture-based testing [85]. Clarithromycin-resistant genotypes can also be detected in stool samples using real-time PCR methods; however, the sensitivity is lower [87]. Real-time PCR assays have also been developed to detect point mutations in the quinolone resistance-determining region of the *gyrA* gene resulting in resistance to ciprofloxacin and point mutations in the 16S rRNA gene conferring decreased susceptibility and resistance to tetracyclines [88, 89]. The assay for determining fluoroquinolone resistance identifies mutations using two hybridization biprobes designed to detect the most frequently occurring mutations at amino acid positions 87 or 91 [88]. Tetracycline resistance is detected using 16S rDNA primers and a fluorescently labeled probe complementary to the wild-type 16S rDNA allele. In both assays, melting curve analysis differentiates amplicons with resistance mutations from those with wild-type sequences [89]. While various mutations in the NADPH nitroreductase gene (*rdxA*) are associated with metronidazole resistance, detection of these mutations is not a reliable indicator of resistance [90].

Histopathologic diagnosis of *H. pylori* infection is a sensitive and specific method (>95 % and 100 %, respectively) under optimal conditions, yet ancillary molecular techniques such as FISH may help in difficult cases [56]. Visualization of the characteristic bacterial forms may be difficult when reduced numbers of bacteria are present, such as when biopsies are obtained after eradication therapy or if the patient has been on long-term acid suppression therapy with PPIs. These same conditions may change the typical morphology of *H. pylori* from a comma or S-shaped bacillus to a coccoidal form, obscuring a visual diagnosis. Several studies using fluorescently labeled, species-specific probes have demonstrated the ability of FISH to reliably detect *H. pylori* [91, 92]. Additionally, clarithromycin-resistant strains also can be detected using FISH performed on formalin-fixed tissue sections [92]. Fluorescent-labeled oligonucleotide probes designed to detect the most common mutations determining clarithromycin resistance are both sensitive and specific when compared to culture-based susceptibility testing [91]. FISH testing, however, may produce results more rapidly than culture.

Interpretation of Results

PCR tests can achieve equal or better performance when compared to non-molecular tests [58–62]. The sensitivity is highly dependent on the target gene and is discussed in greater detail in the "Laboratory Issues" section below.

The specificity of different PCR test methods varies and determining specificity is complicated by the lack of gold standard. Real-time PCR assays applied to tissue biopsies have detected *H. pylori* at low densities that were missed by histology, UBT, and RUT suggesting that the poorer sensitiv-

ity of these non-molecular assays is due to low numbers of organisms [61]. However, due to the high sensitivity of PCR-based methods and the amplification of DNA from nonviable organisms, isolated positive PCR results in the post-treatment period must be interpreted with caution. Positive results in this setting may represent continued presence of organisms at low levels or nonviable organisms, and, therefore, PCR may not have utility in determining eradication failures in the post-treatment period. Isolated positive PCR results in untreated patients may reflect true infection with a low *H. pylori* density, but may also be due to nonspecific amplification of non-*H. pylori* bacterial DNA [93]. Due to these factors, PCR-based methods should not be used as the sole diagnostic test.

Like PCR testing of biopsy specimens, the specificity of results obtained from PCR testing of stool samples may be decreased due to amplification of nonviable organisms. Studies examining the use of PCR-based testing of stool for determining eradication success rates in the early post-treatment period are conflicting [80, 94]. While negative PCR results within 12 days of treatment were obtained for a small group of infected patients, another study demonstrated false-positive results occurring in half the patients 1 month after treatment [94, 95]. False-positive PCR results decrease after longer follow-up periods, and approach zero after 12 weeks of therapy [94]. In addition, analytical specificity of PCR testing on stool samples may be reduced due to the presence of non-*pylori Helicobacter* species present in fecal material [56]. While clinical specificity for PCR-based assays is determined by comparison with UBT, culture, and RUTs, determinations of analytical specificity by testing of non-*pylori Helicobacter* species is rarely performed [96].

Antibiotic resistance genotype testing using real-time PCR can produce results indicating the presence of more than one genotype [85]. These results are interpreted as representing a mixed population and combinations of one or more distinct mutant strains among wild-type strains have been detected [85]. While some studies have detected mutant strains in the presence of wild-type strains down to a level of 10 % [85], other studies cite failed resistance detection due to high levels of susceptible strains [87].

Laboratory Issues

Sensitivity and specificity of PCR-based methods are greatly dependent on primer choice and target gene. Additionally, significant inter-study variation in sensitivity and specificity exists for several of the commonly used primers. Nucleotide differences among distinct *H. pylori* strains may partly explain this test performance variability [77]. A study comparing the diagnostic performance of several different primers demonstrated poor specificity for SSA gene primers and unsatisfactory sensitivity for the *ureA* gene and random *H. pylori* genome sequences [76]. While this study concluded that *glmM* gene PCR performed best, other studies have reported lower specificities [60, 77]. Assays using 16S rRNA sequences generally report excellent sensitivities, but the specificity of these primers is questionable. Several authors argue that 16S rRNA primers are inappropriate because of sequence conservation among different bacterial genera as well as the possibility for nonspecific amplification of human DNA [76, 77]. Assays targeting *vacA* have reported moderate sensitivity (89.5 %) but excellent specificity (99.0 %) [84]. The HSP60 gene is thought to be both well conserved and demonstrates species-specific variation [77]. A nested assay design using primers targeting HSP60 claims to have sensitivity and specificity approaching 100 % [77].

Determining whether tissue to be used for PCR assays is preserved by formalin fixation or cryopreservation represents an important variable in testing, but may ultimately be decided by proximity of laboratory and endoscopy suite. Both formalin-fixed and frozen tissue specimens may be used for PCR-based testing, although frozen samples are far superior [56]. Formalin fixation causes DNA to fragment; however, assays using formalin-fixed tissues may still perform acceptably if short DNA sequences are targeted.

PCR assays applied to stool specimens suffer from inconsistent results attributable to substances inhibitory to PCR amplification, low numbers of *H. pylori* organisms within fecal samples, as well as degradation of DNA during intestinal transit [53, 56]. To avoid false-negative results, complex purification and extraction steps to eliminate PCR inhibitors are required before DNA amplification. Performance of the different biochemical, immunologic, and physical purification methods varies due to degradation of target DNA and incomplete removal of inhibitors [96]. Frozen stocks of *H. pylori* strain NCTC 11637 and dried genomic DNA from that strain (American Type Culture Collection 43504D, Manassas, VA) as well as titered cultures of *H. pylori* (ZeptoMetrix Corp, Buffalo, NY) are commercially available reference materials. Proficiency testing exercises are not commercially available.

Viral Agents

The most common causes of viral gastroenteritis include adenovirus serotypes 40 and 41, rotavirus, astrovirus, and caliciviruses (noroviruses, sapoviruses). Conventional detection of these viruses is based on antigen detection and EM. Molecular methods have been primarily used for epidemiologic or research purposes but have also demonstrated significant improvement in the diagnosis of viral gastroenteritis and are becoming available in clinical laboratories.

Other viruses less frequently implicated as causes of acute gastroenteritis include coronaviruses and toroviruses.

In addition, viruses such as cytomegalovirus and herpes simplex virus are opportunistic causes of enteric disorders in patient infected with the human immunodeficiency virus (HIV) or with compromised immunity but are diagnosed by examination or testing of gastric or intestinal biopsy tissues rather than examination of stool.

Adenovirus

Description of Pathogen

Adenoviruses are nonenveloped viruses with a linear, nonsegmented, double-stranded DNA genome surrounded by an icosahedral protein capsid. The genome size varies among adenoviral groups and is between 26,000 and 45,000 nucleotides which theoretically provides the capacity for 22–40 genes. The genome consists of immediate early (E1A), early (E1-E4), intermediate, and late genes (L1-L5).

Adenoviruses are classified into five genera including Atadenovirus, Aviadenovirus, Ichtadenovirus, Mastadenovirus, and Siadenovirus. The adenoviruses that infect humans belong to the genus *Mastadenovirus*. Seven species of human adenovirus (A through G) within the genus *Mastadenovirus* are currently recognized. Species designations are determined by immunologic properties as well as DNA homology and oncogenicity [97]. Each species group contains several serotypes classified by neutralization reactions to specific antisera [97]. At present, over 50 serotypes have been described [98]. Serotype groups may be further subdivided into genomic types. Genotypes are assigned lowercase letters to differentiate them from the prototype strain as indicated by the letter "p" [97]. Interspecies DNA sequence variation may be as low as 4 % whereas genotypes within an adenovirus species may be 50 % to nearly 100 % homologous [99, 100]. Intraspecies recombination resulting in intermediate strains has been reported [101].

Species F serotypes 40 and 41 are the most frequent adenovirus serotypes isolated from patients with gastroenteritis and are referred to as the "enteric adenoviruses." These serotypes are second only to rotavirus as the most common cause of acute diarrheal illness in children [102]. Adenoviruses of all serotypes are implicated in approximately 5–15 % of childhood diarrhea cases [97]. Gastroenteritis due to adenovirus occurs worldwide and the incidence does not demonstrate significant seasonal variation [103]. More than one serotype or species may be isolated in a given patient [104]. Serotypes infrequently associated with gastroenteritis include 1, 2, 3, 5, 7, 12–18, 21, 25, 26, 29, 31, and 52 [102].

Transmission is thought to occur by fecal-oral spread and the mean incubation period for gastroenteritis is 3–10 days [97, 103]. After clinical symptoms improve, enteric adenoviruses are shed in stool rarely for longer than a few days compared to patients with respiratory infections not involving enteric adenoviruses who may shed for 3–6 weeks and as long as 18 months [97]. The duration of viral shedding in the gastrointestinal tract may be prolonged in immunocompromised individuals [97]. Adenoviruses can also be shed in the stool of individuals with asymptomatic infections which are common, particularly in children [97].

Clinical symptoms include watery, non-bloody diarrhea accompanied by mild fever, vomiting, and abdominal pain. Gastroenteritis in immunocompetent patients usually resolves without complication; however, rare fatalities are documented [97].

Gastrointestinal infections in immunocompromised patients occur most often in hematopoietic stem cell (HSCT), bone marrow (BMT), and solid organ (SOT) transplant patients. Infections in these patients are frequently severe and can become disseminated. In pediatric allogeneic HSCT patients, detectable virus in stool almost always precedes systemic adenovirus infection [105]. Adenovirus species isolated most frequently in HSCT and BMT patients with gastrointestinal disease include species A (serotype 31), B (serotype 7), and C (serotype 2) [106]. The incidence of adenovirus infections in patients with HIV infection or acquired immunodeficiency syndrome (AIDS) has dropped due to effective treatment of the HIV infection with highly active antiretroviral therapy [10]. Serotypes within species D cause the majority of gastroenteritis in HIV-positive patients and include serotypes 9, 17, 20, 22, 23, 26, 27, and 42–51 [106].

Clinical Utility of Testing

Diagnosis of adenovirus gastroenteritis is primarily determined through testing of stool samples although tissue biopsy specimens also may be used. Conventional methods used to identify the presence of adenovirus in stool samples include shell vial cultures, direct fluorescent antibody assays, EIAs, and EM. Disadvantages of culture methods include delays of up to weeks and false-negative results with difficult to culture AV serotypes such as 40 and 41 [99]. Immunofluorescent and immunochromatographic methods, while rapid, are insensitive [107]. EM also is insensitive and is not routinely used in clinical laboratories. Indirect diagnosis using serology is limited by poor sensitivity especially in immunocompromised patients and by high seroprevalence among children preventing the ability to identify acute disease [107, 108]. Despite these diagnostic limitations, conventional methods may be sufficient to detect infection in immunocompetent patients with localized or benign gastrointestinal symptoms [109].

Adenovirus infection may become severe in immunocompromised patients and the ability to begin early treatment, such as reduction of immunosuppression or cidofovir therapy, requires rapid and sensitive diagnostic techniques [107]. Several PCR-based assays have been validated for

stool specimens and are comparable or better than conventional methods [110–112]. Both qualitative and quantitative PCR assays are used. Qualitative assays vary in serotype detection of all or only some serotypes. While qualitative PCR methods are sensitive, quantitative assays assess stool viral load and proliferation kinetics. These parameters may become important in assessing the need for preemptive treatment of adenovirus infection in pediatric HSCT patients by detection of significant levels of virus in stools before the onset of viremia and disseminated infection in the majority of these patients [105, 113].

Multiplex assays differ in their clinical application. One particular assay provides qualitative, yet species-specific results [114], while others offer quantitative results and detect adenovirus in combination with other important gastrointestinal pathogens. Quantitative assays may be useful for determining which pathogen is responsible for disease in cases of mixed infections [115, 116].

In most cases, determination of adenovirus serotype is unnecessary for clinical management [117]. By contrast, serotyping is important for epidemiology studies, when investigating an especially severe infection, and in predicting clinical outcome [106]. Isolation of specific adenovirus serotypes from the gastrointestinal tract, such as 1, 2, 5, and 6, may raise concern for the possibility of disseminated disease since these serotypes have been documented to cause systemic infections in immunocompromised patients [107]. On the other hand, serotypes 40 and 41, while frequently isolated in cases of gastroenteritis, have not been recovered from immunocompromised patients with disseminated infections [107]. While conventional serotyping methods may take up to several weeks [97], molecular methods such as PCR have improved turnaround times and allow for the characterization of isolates at the species, serotype, and genotype level [107]. Molecular and serological typing results usually are concordant [97].

Available Assays

Tissue biopsies may be submitted for culture or for histological examination using hematoxylin-and-eosin or Wright-Giemsa stains and immunohistochemistry. Molecular techniques such as in situ hybridization may aid diagnosis by confirming characteristic microscopic findings [118]. Alternatively, biopsy specimens may be submitted directly for molecular testing using conventional or real-time PCR [102, 119].

Molecular methods offer increased sensitivity and shorter turnaround time compared with conventional methods [110–112]. Different PCR methods for the detection of adenovirus in stool or tissue biopsies include conventional PCR, real-time PCR, and multiplex PCR. Most clinical laboratories use LDTs because no US FDA-cleared tests are available for stool testing. Regardless of the PCR method, degenerate or non-degenerate primers and probes targeting the hexon or fiber genes or the VA RNA-encoding regions are typically used. These regions display homology across serotypes for consistent binding of primers and probes, yet also include hypervariable regions suitable for differentiating serotypes.

Conventional PCR assays range in their detection abilities. Some systems detect and report specific serotypes [110, 120, 121], while others report genus- or species-specific results and purport to detect all serotypes [107, 117]. These assays are qualitative and usually have a 1–2 day turnaround time. Methods used to detect PCR amplicons include ethidium bromide-stained gel electrophoresis, Southern blotting, or liquid phase hybridization quantitated by time resolved fluorometry [122]. These detection methods are time- and labor-intensive and necessitate handling of PCR products, thus potentiating the risk of contamination.

Real-time PCR methods offer quantitative results and are more rapid and involve less contamination risk than conventional PCR assays [102, 119, 123, 124]. Several LDTs and one commercial assay are validated for use on stool specimens. While some methods rely on a single probe and primer pair, most utilize more than one set of primers and multiple probes. Weighing of stool specimens before DNA extraction allows results to be quantitated in copies per gram of stool. This standardization permits assessment of serial stool specimens for viral load kinetics and facilitates comparison of results between assays.

Several multiplex PCR assays have been validated for stool testing and differ in their clinical applications and detection methods. One particular method allows for identification of all six adenoviral species in a single reaction mixture using species-specific hexon primers [110]. Species-specific results are visualized by agarose gel electrophoresis, which shows a different amplicon length for each species [110]. Other multiplex assays offer quantitative results and combine adenovirus detection with other common gastrointestinal viral pathogens [115, 116]. Detection techniques differ and involve either fluorescent-labeled sequence-specific probes or sequence-specific capture probes bound to microspheres, which are interrogated by flow cytometry.

Conventional typing may take weeks making such methods impractical for clinical use. Molecular typing methods greatly improve turnaround time and several assays have been tested for use with stool samples. Strategies for producing serotype or genotype specific results vary by assay, and may be performed from cultured isolates or directly from clinical specimens [125, 126]. Traditional molecular typing methods rely on REA and may be performed on adenoviral genome DNA or following PCR amplification of specific regions [117, 127]. Genotype or serotype is inferred from the band pattern on agarose gel electrophoresis. REA methods are still used to identify new strains or for type identification

of an isolate causing severe disease [106]. Sequence-based typing may be used to determine both serotype and genotype and is usually performed after PCR amplification of hypervariable regions [128]. Generated sequences are compared to banked sequences of known serotypes.

Interpretation of Results

Similar to other clinical situations where highly sensitive molecular assays are applied, PCR methods used in the diagnosis of adenovirus gastroenteritis offer improved sensitivity over conventional methods, but may provide positive results in the absence of disease. The ability for adenoviruses to cause asymptomatic infection and the tendency for nonenteric adenoviruses to be shed in the stool for weeks to months after resolution of clinical symptoms make interpretation of positive results in patients without symptoms difficult [97, 103].

Diagnosis of adenovirus gastroenteritis in immunocompetent patients is straightforward when PCR results are positive for an enteric adenovirus species or serotype in the presence of characteristic symptoms. Viral shedding in the absence of symptoms is unusual for enteric adenoviruses, especially in immunocompetent individuals.

Shedding of nonenteric serotypes for long periods of time occurs more frequently in immunocompromised patients and makes it difficult to determine conclusively that a detected adenovirus serotype is the cause of the patient's symptoms. Persistent viral shedding from a previous adenovirus infection may be difficult to distinguish from a newly acquired asymptomatic infection, which occurs often in the immunocompromised patient population. Further complicating interpretation in immunocompromised individuals is the frequent occurrence of coinfections.

The ability to determine serotype by sequencing is limited by incomplete reference databases containing sequence information for only certain serotypes [107]. The completeness of the reference database depends on the genome region sequenced. Serotype determination by methods relying on enzyme restriction patterns is limited to serotypes whose restriction patterns have been previously described. These methods are further hampered by the genetic variability created over time by recombination events between viruses of different serotypes. Such variation may alter cleavage sites and create unrecognizable restriction enzyme patterns. Infections caused by one or more serotypes may also create uninterpretable results.

As mentioned above, quantitative testing of stool allows clinicians to monitor stool viral load and proliferation kinetics. The mere presence of adenovirus in stool is common in pediatric HSCT patients, and does not necessitate treatment, as the majority will clear the virus spontaneously [129]. Quantitative measurements, however, have allowed investigators to identify rising stool viral loads in the majority of pediatric HSCT patients who go on to develop adenovirus viremia and disseminated disease [105, 113]. Quantitative serial stool measurements, therefore, may serve as a useful tool for predicting when early treatment is warranted and could prevent the overuse of the nephrotoxic antiviral drug cidofovir [113].

Laboratory Issues

Detection of all adenovirus serotypes is important because serotypes other than enteric adenoviruses cause a significant number of gastroenteritis cases in immunocompromised patients. The high degree of genetic heterogeneity among adenovirus serotypes makes detection of all known serotypes by a generic PCR assay difficult [106]. Genetic diversity also complicates identification of regions with sufficient homology to allow for uniform annealing of primers and probes in all serotypes. Currently, most assays use primers and probes that bind to the highly conserved hexon gene, which has only approximately 50 % nucleotide homology between serotypes (NCBI database, [105]). Uniform annealing of primers and probes is even more important for quantitative assays, to ensure equal amplification efficiency of all serotypes [108]. Concern that nucleotide mismatches between target and primer or probe would result in decreased sensitivity of detection for many serotypes has led to more optimal assay designs utilizing multiple primer and probe sets [123]. Lastly, multiplex assays that detect multiple viral pathogens can detect coinfections, although the test performance for adenovirus was occasionally negatively affected by coamplification of other viruses [116].

DNA from both adenovirus 40 and adenovirus 41 is available from ATCC (Manassas, VA). The Zeptometrix NATtrol™ gastrointestinal pathogens verification panel includes adenovirus among the other analytes. The Stool Pathogen Panel (SP) proficiency survey from the College of American Pathologists includes challenges for adenovirus 40/41.

Rotavirus

Description of Pathogen

Rotaviruses are non-enveloped viruses in the *Reoviridae* family, named because of their characteristic wheel-like appearance by EM. Rotaviruses are very stable in the environment and can remain infectious for several weeks. They have a triple-layered structure with concentric capsid layers that surround a core which contains the genome. The surface of the outermost capsid layer contains two major structural viral proteins, VP4, a protease-cleaved protein (P protein), and VP7, a glycoprotein (G protein). The middle

layer of the capsid contains structural protein VP6. The inner capsid layer contains proteins VP1, VP2, and VP3. The rotavirus genome consists of 11 segments of double-stranded RNA with a complete genome length of 16,500–21,000 nucleotides. The genomes can reassort during dual infection of a single cell which results in co-circulation of a wide variety of strains.

Rotaviruses are classified into serogroups A through G based on the antigenic characteristics the VP6 protein. Only groups A, B, and C infect humans and animals. The remaining rotavirus groups have been found only in animals. The Group A human rotaviruses cause the majority of viral gastrointestinal infections in children. Group B rotaviruses were first identified as causing adult diarrhea in a large waterborne epidemic in China. Serologic evidence indicates that Group B rotavirus is also present in the UK and the US, and genome profiles consistent with Group B rotavirus have been detected as causes of diarrhea in India [130]. Group C rotaviruses are an emerging cause of gastroenteritis in both children and adults and have been identified in sporadic cases and outbreaks worldwide. The Group A rotaviruses are further classified into serotypes based on neutralizing serologic reactions against the P (VP4) and G (VP7) proteins. Also, because the two gene segments that encode the P and G proteins segregate independently, a genotyping system has been developed based on the sequences of both genes. The most prevalent Group A rotavirus genotypes in humans are G1P[8], G2P[4], G3P[8], G4P[8], and G9P[8]. A Rotavirus Classification Working Group has been formed to assist in classification of any newly described rotavirus genotypes based on sequence information for all 11 genomic RNA segments.

Rotavirus is endemic worldwide and is the single most common cause of diarrhea among infants and young children [131]. Most rotavirus infections are self-limiting but some children become very ill with severe vomiting, diarrhea, and life-threatening loss of fluids that requires hospitalization. Death due to rotavirus infection is relatively rare in the USA but is a significant concern in developing countries. Rotaviruses are estimated to cause more than half a million infant and young children deaths worldwide every year [131].

In the USA and other countries with temperate climates, annual outbreaks of rotavirus infection occur during winter and spring, with fewer cases in summer. However, with the introduction of rotavirus vaccines, the seasonality has shifted and the winter–spring trend in peak rotavirus activity is no longer consistently observed [132]. Seasonal variation is not seen in tropical climates.

Rotaviruses are shed in large quantities in the stools of infected children beginning 2 days before the onset of diarrhea and for up to about 10 days after the onset of symptoms. Immunocompromised individuals may shed detectable rotavirus for more than 30 days after infection. Rotaviruses are highly communicable, with a small infectious dose of less than 100 virus particles [133]. Rotaviruses are spread by fecal-oral transmission, both through close person-to-person contact and through fomites, and are common causes of diarrheal outbreaks in families, in childcare centers, and other institutions, and among hospitalized children [134]. The incubation period for rotavirus illness is about 2–3 days. Immunity after infection is incomplete, but repeat infections tend to have milder signs and symptoms than the initial infection.

A rotavirus vaccine is now included in the American Academy of Pediatrics recommended immunization schedule for infants. Two vaccines, RotaTeq® (Merck & Co., Inc., West Point, PA) and Rotarix® (GlaxoSmithKline, London, UK) were introduced in 2006 and 2008, respectively, and are currently licensed for use in the USA. A previous rotavirus vaccine was taken off the market in 1999 because of an increased risk for intussusception, which does not occur with either RotaTeq or Rotarix.

Adults and older children also can be infected with rotaviruses. Infection in adults is often subclinical or very mild. Clinically evident cases are most often seen in immunocompromised patients, the elderly, and travelers to developing countries [135].

Clinical Utility of Testing

Rotavirus infection cannot be diagnosed by clinical presentation because the clinical features of rotavirus gastroenteritis do not differ from those of gastroenteritis caused by other pathogens. Confirmation of rotavirus infection by laboratory testing is used for surveillance but also is useful in clinical settings to avoid inappropriate use of antimicrobial therapy.

Since rotavirus is present in high concentrations in the stool of infected children, stool is the preferred specimen for diagnosis. Rotaviruses can be cultured in Madin-Darby bovine kidney (MDBK), fetal African green monkey kidney cells (MA104 cell line), and some other cell lines in media containing trypsin or pancreatin, but culture is relatively inefficient and not performed in clinical laboratories. The most widely available method for detection of rotavirus antigen in stool is EIA directed at the VP6 antigen common to all group A rotaviruses. Several commercial EIA kits are available, which are inexpensive, easy to use, rapid, and sensitive (approximately 90–100 %). Latex agglutination is less sensitive and specific than EIA but is still used in some settings. Immunochromatographic point of care tests have reported sensitivities of 94–100 % and specificities of 96–100 % compared with clinical laboratory tests [136]. Other techniques, including EM, reverse transcription-polymerase chain reaction, nucleic acid hybridization, sequence analysis, and culture are used primarily in research settings.

Molecular methods have been used primarily for characterization of G and P genotypes in epidemiologic studies

and to evaluate the impact of vaccination [137, 138]. RT-PCR has increased detection rates for rotavirus A by up to 48 % compared to EIA or EM [139]. Sensitivity of RT-PCR tests is estimated at 10^4 rotavirus particles per milliliter of stool, while EIA methods detect 10^6 rotavirus particles per milliliter of stool. While increased sensitivity is usually seen as a benefit, some have considered RT-PCR assays too sensitive for the detection of rotaviruses due to their ability to detect asymptomatic infections, which are common in infants and young children [140, 141].

Available Assays

Some commercial tests designed for testing food sources or environmental samples are available as research use only kits. Most reports of molecular tests used for diagnosis of human rotavirus infections are LDTs. The xTAG GPP gastrointestinal pathogen panel test kit (Luminex Corp., Austin, TX) is an US FDA-cleared qualitative RT-PCR multiplexed test that can be performed in about 5 h and simultaneously detects the most common parasitic, bacterial and viral gastrointestinal pathogens, including rotavirus Group A.

Interpretation of Results

Asymptomatic carriage of rotavirus can be detected by molecular tests and needs to be considered when interpreting positive results in clinical specimens [140, 141]. Asymptomatic carriage vs true mixed infection also needs to be considered in the rare event that rotavirus is detected in combination with another gastrointestinal pathogen in the same clinical sample or during an episode of diarrhea. Laboratories should be aware that rotaviruses can be detected by RT-PCR in clinical specimens for about 10 days after resolution of an acute diarrheal episode in healthy children [137].

Laboratory Issues

Ideally, diagnostic RT-PCR tests would be able to detect all three genogroups of human rotaviruses. Except for epidemiologic purposes, identification of the specific genogroup is probably not necessary. Laboratories should be aware that rotavirus RNA has been detected in serum, CSF, and throat swab specimens [142, 143].

The NATtrol™ (ZeptoMetrix Corp, Buffalo, NY) verification set includes reference material for rotavirus among other analytes. Human rotavirus in diarrheal stool samples is available from ATCC (Manassas, VA). The Gastrointestinal Panel for Molecular Multiplex Testing (GIP) and the Stool Pathogens (SP) survey, both from the College of American Pathologists, include proficiency testing challenges for rotavirus among other gastrointestinal pathogens.

Astrovirus

Description of Pathogen

Astroviruses are small, round, non-enveloped viruses with icosahedral cubic capsids that have a characteristic five or six-point star-like surface structure when viewed by EM. Astroviruses contain three structural proteins (VP26, VP29, and VP32). The genome is composed of non-segmented, positive-sense, single-stranded RNA. The total genome length is 6,800–7,900 nucleotides, excluding the poly (A) tract at the 3′ end and the genome has been sequenced.

The family *Astroviridae* contains two genera: *Mamastroviruses* which infect numerous types of mammals and *Avastroviruses* which infect birds (e.g., ducks, chickens, turkeys). Within each genus are species of astroviruses, which, according to International Committee on Taxonomy of Viruses guidelines, are named based on the host in which they replicate. The astroviruses are further subclassified within each species into serotypes. Three species of astroviruses are found in human stool: HAstV (serotypes 1–8), AstV-MLB, and HMOAstV (serotypes A to C). These species are more closely related to animal astroviruses than to each other, indicating phylogenetically separate origins of human astroviruses [144].

Simultaneous circulation of multiple types of astrovirus is not rare [144]. Human astrovirus serotype 1 (HAstV-1) is the most prevalent serotype detected worldwide. However, serotype 3 produces higher quantities of virus in stool and appears to cause a larger proportion of cases of persistent gastroenteritis [145].

Human astroviruses are endemic worldwide. Studies using sensitive detection techniques, such as RT-PCR, have demonstrated that astrovirus infection is a more common and important cause of viral gastroenteritis than previously known. Symptomatic illness is most common in children <2 years of age, although infection in immunocompromised individuals and outbreaks among adults and the elderly have also been reported. Astroviruses are highly stable in the environment and are resistant to a wide range of detergents and lipid solvents. The fecal-oral route is thought to be the most common means of transmission and contaminated food, water, and fomites are common sources of virus. As with many other viral causes of gastroenteritis, astrovirus infection has a peak incidence in winter in temperate climates and is associated with the rainy season in tropical regions. Astrovirus is thought to replicate in the intestinal tissue of the jejunum and ileum and generally causes mild, self-limiting illness of short duration. Prevalence is likely under-estimated since surveillance and seroprevalence studies have demonstrated that astrovirus infection is common and is largely asymptomatic.

Immunity to astrovirus infection is not well understood. Prevalence of symptomatic infection among young children and institutionalized elderly populations suggests that antibody is acquired early in childhood, provides protection through adult life, and begins to decline later in life [146]. Heterologous protection does not occur across the human astrovirus serotypes [147].

Clinical Utility of Testing

No vaccine or anti-viral treatment is available for prevention or treatment of astrovirus infection, but diagnosis may be important to avoid unnecessary antibiotic use. Establishing an etiology also may be important in hospitalized patients for infection control purposes to prevent nosocomial spread [148, 149]. Further, diagnosing astrovirus gastroenteritis in patients with malnutrition, immunodeficiency, and underlying gastrointestinal disease, may be important because of the increased likelihood of complications that require hospitalization in these populations. The impact of astrovirus infection on the morbidity of infants and children may become increasingly important as the rotavirus vaccine becomes more widely used and the burden of rotavirus is reduced.

Available Assays

Astroviruses have been adapted to cell culture using CaCo-2 human colonic carcinoma cells in some research settings, but are not cultured for clinical diagnostic purposes. EM and immune EM (IEM) have been effectively used to detect astroviruses in clinical stool specimens but are not available in many clinical laboratories. Also, identification by EM can be difficult since only a small portion of astrovirus particles (about 10 %) display the characteristic star-like morphology [150].

EIA tests have been developed for the qualitative detection of astrovirus antigen in clinical specimens and are commercially available in Europe (e.g., RIDASCREEN® Astrovirus test, R-biopharm AG, Darmstadt, Germany, IDEIA™ Astrovirus, Dako Diagnostics Ltd, Ely, UK) but are not US FDA-cleared for diagnostic use in the USA.

Commercial real-time RT-PCR kits are available for testing environmental and food samples. Molecular assays are considered to be an improved diagnostic method over EM and EIA [151], but US FDA-cleared diagnostic tests for astrovirus are not available. Specifications for LDTs for detection of astrovirus in clinical samples using highly sensitive group-specific RT-PCR primers targeted to conserved genomic regions coding for the nonstructural proteins and untranslated regions are available [151]. Reported detection limits for these assays vary from 1 to 10 viral copies depending of the quality of the analyzed nucleic acid. Some tests utilize primers from the capsid coding region which can be less sensitive, but provide type information [151].

Interpretation of Results

Shedding of astrovirus is generally limited to about 1 week in immunocompetent individuals, but as with other viruses that cause gastroenteritis, prolonged shedding of astrovirus (e.g., 4 weeks) has been observed in immunocompromised patients [148, 152]. Although asymptomatic infection is common, determining the significance of astrovirus detection should not be problematic in most clinical settings since presumably only diarrheal stools from symptomatic patients would be tested.

Laboratory Issues

Astrovirus infections are generally limited to the gastrointestinal tract; however, astroviruses have been detected in plasma as a cause of febrile illness and in brain tissue of an immunocompromised patient [153]. Human gastrointestinal astrovirus infections have been limited to the eight closely related serotypes described above. Recently, several highly divergent astrovirus serotypes (MLB1, MLB2, VA1, VA2, and VA3) have been detected in stool samples from patients with and without diarrhea [153]. An association with gastrointestinal disease has not been definitively made for these newly described astroviruses [153]. Reference material and proficiency testing challenges are not currently commercially available.

Caliciviridae (*Noroviruses, Sapoviruses*)

Description of Pathogen

Human caliciviruses belong to the family *caliciviridae* and are small, round, non-enveloped viruses with a single-stranded, positive-sense RNA genome. The family currently includes the genera *Norovirus* (previously Norwalk and Norwalk-like viruses), *Sapovirus* (previously Sapporo and Sapporo-like viruses), *Lagovirus*, *Vesivirus*, and the newly proposed *Becovirus* and *Recovirus*. The noroviruses and sapoviruses have recognized roles as causes of acute gastroenteritis in humans. Within each genus, strains are further grouped into genogroups and genotypes or clusters.

The noroviruses are partitioned into genogroups GI to GVII, each further subdivided into genotypes and subgenotypes. Porcine, bovine, and murine noroviruses belong to genogroups II, III, and V, respectively. The majority of human norovirus outbreaks are caused by genogroup II genotype 3 (GII-3) and genogroup II genotype 4 (GII-4) viruses. Human norovirus are thought to be specific to humans and transmission from an animal reservoir has not been described. However, at least three clusters of porcine noroviruses in genogroup II are genetically closely related to the human noroviruses in genogroup II, introducing the potential for zoonotic transmission [154].

The sapoviruses are similarly partitioned into five genogroups (GI to GV) with genotypes in each group. Human sapoviruses belong to genogroups GI, GII, GIV, and GV. GIII contains the porcine strains. Caliciviruses appear to naturally undergo recombination during normal replication of the virus, leading to the emergence of a continuous array of new variants [155].

Human caliciviruses are cannot be grown in standard in vitro cell culture assays and their role as agents of gastrointestinal diseases was under appreciated because clinical tests for the detection of caliciviruses were not commonly available. Molecular methods such as RT-PCR have revealed that caliciviruses are broadly distributed worldwide and are very common causes of epidemic and sporadic gastroenteritis in both children and adults [156]. The study of noroviruses is significantly more advanced than that of sapoviruses. Noroviruses are recognized as the leading cause of epidemic gastroenteritis, often causing large water- or food-borne outbreaks in all ages, while sapoviruses are implicated mainly in pediatric gastroenteritis [157].

Caliciviruses are presumed to replicate primarily in the upper intestinal tract and histopathologic lesions are seen in the jejunum of infected individuals. Symptoms of calicivirus infection are popularly known as "stomach flu" and include vomiting, abdominal cramps, diarrhea, headache, and fever. Symptoms generally last 1–4 days and most people recover completely without treatment. Infants, older adults and people with underlying disease can become severely dehydrated and require medical attention. Asymptomatic infections with shedding of virus are common, and could be the source of some outbreaks [158]. Protective immunity is thought to be short-lived and individuals who have been infected may or may not be immune to reinfection. Studies of immune response are complicated by the ability of the virus to produce naturally occurring variants which are difficult for the immune system to recognize.

Caliciviruses are stable in the environment and can survive freezing, heating to 60 °C, and in chlorinated water up to 10 ppm. They can also survive for several days on many types of surfaces (e.g., door knobs, counter tops, pens, and telephones). Caliciviruses are highly contagious with an estimated infectious dose as low as 10–100 virus particles [159]. These characteristics facilitate rapid spread of caliciviruses, especially in households and institutional settings such as schools, day care centers, hospitals, nursing homes, restaurants, and cruise ships [160]. Calicivirus infections occur year round, although a winter seasonal peak is frequently observed [161].

Transmission is thought to occur mainly through fecal-oral routes. Evidence also suggests that caliciviruses may be transmitted by close exposure to aerosols generated during vomiting episodes in infected individuals. The most frequent cause of norovirus infection appears to be consumption of food or beverages that are contaminated either at their source or by infected food handlers. Uncooked shellfish, particularly oysters, as well as raspberries and precooked foods, such as salad, ham, and sandwiches, are among the common foods that have been responsible for outbreaks [161]. Outbreaks resulting from contamination of municipal water are rare, but water-borne outbreaks associated with community or family water systems have been documented. Sapoviruses have been associated with food-borne outbreaks, but much less frequently than noroviruses. Sapovirus infections are not associated with eating seafood. Nosocomial infections due to caliciviruses are increasingly recognized and may be quite common.

Clinical Utility of Testing

Laboratory diagnosis of calicivirus infection is difficult and clinical diagnosis is often used, especially when other agents of gastroenteritis have been ruled out. Detection of caliciviruses may be important because of their biologic, physicochemical, and epidemiologic features, which present significant challenges for infection control in hospital environments. The regular turnover of patients leaving the hospital and being replaced by new patients provides an opportunity for introduction of the virus from the community and subsequent transmission within the hospital environment.

Available Assays

Although Norovirus has been adapted to grow in a complex 3D culture system in research settings, attempts to culture human caliciviruses in routine cell lines have failed to yield replicating virus, even with addition of a wide variety of culture supplements.

Caliciviruses can be detected fairly rapidly in stool specimens from patients with diarrhea using direct EM. Detection by direct EM requires virus concentrations of at least 10^6 virus particles per milliliter of stool, making EM relatively insensitive, even after processing of the specimen to concentrate the virus particles [158]. IEM improves the sensitivity of direct EM by 10- to 100-fold, but this technique is infrequently used. False-negative results can occur if antibody is present in excess and masks detection of the virus [160]. Use of EM is limited due to the need for expensive equipment, experienced technologists and a significant amount of labor per specimen [162].

The cloning of the Norwalk virus genome and subsequently of other human caliciviruses has allowed the development of other methods for diagnosis. EIAs to detect virus in stool specimens using polyclonal hyperimmune animal sera can detect the presence of 10^4–10^6 intact virus particles/ml of stool [160, 163], but are considered to have inadequate

sensitivity to be used for diagnosis, especially of sporadic cases [164]. Serologic EIAs to detect specific antiviral antibodies generally are used to detect a fourfold increase in antibody levels between acute and convalescent serum specimens. The hyperimmune antisera and recombinant antigens needed to produce these EIAs are not widely accessible and US FDA-approved diagnostic assays have not been produced. The assays are available at some public health laboratories and are primarily used for epidemiologic purposes.

Since caliciviruses cannot be grown in vitro, EM is available only in highly specialized facilities, and serology assays are insensitive, the potential utility of RT-PCR-based techniques is considerable. The highly variable nature of calicivirus genomes presents a significant challenge to designing molecular assays for diagnostic use. The literature contains details of a variety of RT-PCR assays that have been used for the detection of human caliciviruses in diagnostic studies as well epidemiologic investigations of food, water, and other environmental samples. Some of the assays are designed to detect sapoviruses without cross-reactivity with noroviruses or rotaviruses. Among them is a quantitative real-time PCR assay using primers against the junction of the RNA-dependent RNA polymerase/capsid genes that has been described to detect sapovirus genogroups I, II, and IV with an analytical sensitivity of ten copies of viral cDNA per reaction [165]. Conventional qualitative RT-PCR assays that detect all genogroups of human sapoviruses have also been developed [166]. Nested RT-PCR assays that claim to be more sensitive than conventional PCR that detect and differentiate all genogroups of human sapovirus have been reported using primers against the RNA-dependent RNA polymerase region [167] or the capsid-protein coding region [168]. Molecular assays using various protocols and primer sets to detect noroviruses have been similarly described. Only a small region of approximately 50 base pairs at the polymerase (ORF-1)/capsid (ORF2) junction of the norovirus genome appears to be sufficiently conserved to detect all within-genogroup variants [169–171]. Additional assays have been published that use multiple primers in a multiplex format to amplify the capsid region of the respective viral targets to simultaneously detect norovirus genogroups I and II, sapoviruses, and astroviruses [172].

The public health laboratories in all 50 states of the US, are able to test for norovirus RNA by RT-PCR in stool and emesis specimens, as well as environmental samples. The public health laboratories sponsor CaliciNet, a national network that tracks the different sequences of norovirus strains found in clinical and environmental samples to aid in assessment of relationships between strains in epidemiologic investigations and provide identification of emerging strains.

Several CE-marked molecular tests are available in Europe for the specific detection of Norovirus genogroups I and II in stool samples, including the MutaPLEX® Norovirus real-time RT-PCR kit (Immundiagnostik AG, Bensheim, Germany), the RealStar® Norovirus RT-PCR kit (Altona Diagnostics, Hamburg, Germany), the AmpliSens® *Norovirus* genotypes 1, 2-EPh PCR kit (Ecoli s.r.o., Slovak Republic), the SmartNorovirus (Cepheid, Maurens–Scopont, France) and the Xpert® Norovirus (Cepheid, Sunnyvale, CA). The xTAG GPP gastrointestinal pathogen panel test kit (Luminex Corp., Austin, TX and Milan, Italy) is a qualitative multiplex molecular test that uses the Luminex xTAG® Technology and the xMAP® Technology platform to simultaneously detect the most clinically important bacterial, viral, and parasitic gastrointestinal pathogens, including Norovirus types I and II from a single specimen. The xTAG GPP panel is US FDA-cleared for use in clinical laboratories in the USA and also has regulatory approval in Europe and Canada. The BioFire GI Panel (bioMerieux, Durham, NC) fully automated GI panel also includes detection of Norovirus genogroups I and II as well as sapoviruses.

Interpretation of Results

The increased sensitivity afforded by RT-PCR may make interpretation of results confusing, especially during the management of outbreaks. Norovirus can be detected in stool samples before symptoms occur and continue to be detected for a few days to several weeks after symptoms resolve.

Laboratory Issues

Ideally, diagnostic RT-PCR tests would be able to detect and distinguish the clinically significant genera of human caliciviruses. False-negative results may occur due to the sequence diversity of newly emergent variants which existing primer pairs may not detect. Identification of the specific genogroup is helpful for epidemiologic investigations but is probably not necessary in clinical laboratories. Norovirus is most easily detected in stool specimens obtained during the acute phase of illness (48–72 h after the onset of symptoms) when large numbers of virus are present. Norovirus RNA also has been reported in human serum [173] and in CSF [174].

Quantified, synthetic Norovirus GI and GII RNA standards are available from ATCC (Manassas, VA). The NATtrol™ (ZeptoMetrix Corp., Buffalo, NY) verification set contains reference material for noroviruses and sapoviruses in addition to a variety of bacteria and parasites.

The Gastrointestinal Panel for Molecular Multiplex Testing (GIP), as well as the Stool Pathogens (SP) survey, both from the College of American Pathologists, have Norovirus GI/GII as analytes on the panel, but do not currently include sapoviruses.

Parasitic Pathogens

Protozoa and helminth parasites can infest the gastrointestinal tract and are typically shed in the stool. Parasitic infections are prevalent in Central and South America, Africa, and Asia but are much less common in Australia, Canada, Europe, Japan, New Zealand, and the USA. In developed countries, parasitic infections are most frequently encountered among immigrants and travelers returning from endemic regions and occasionally among individuals who have not traveled, particularly those with AIDS or other causes of immunodeficiency.

Description of Pathogens

Many intestinal parasites may need to be considered in the differential diagnosis of gastrointestinal disease depending on the geographic area visited, specific history of exposure, and individual risk. The most common pathogenic protozoan parasites in developed countries are *Giardia lamblia* and *Cryptosporidium hominis*/*C. parvum* and are seen in travelers returning from endemic areas. *Entamoeba histolytica* is a much less common cause of gastrointestinal illness but early diagnosis is important because of the potential to cause dysentery and invasive extraintestinal disease. Many other intestinal parasites such as *Enterobius vermicularis*, *Cyclospora* sp., *Ascaris* sp., *Cystoisospora* sp., microsporidia, *Trichuris* sp., hookworms, *Strongyloides* sp., tapeworms, flukes, and others are associated with acute and chronic illness in the USA, particularly in socioeconomically poor areas and among immunocompromised individuals. Surveillance using molecular assays is causing reconsideration of the role of some parasites such as *Blastocystis hominis*, *Dientamoeba fragilis*, *Entamoeba coli*, and *E. hartmanii* that were previously considered to be associated with harmless asymptomatic infection but are now thought to have the potential to cause symptomatic illness [175].

Clinical Utility of Testing

Gastrointestinal parasitic infections are not common but cause significant morbidity and mortality, particularly in developing countries and in individuals who are immunocompromised or have other underlying medical conditions. Diagnosis of parasitic disease often is delayed when patients present with vague gastrointestinal symptoms or because of the inability of existing diagnostic methods to detect the causative organism when there is irregular morphology, a low parasite load, or intermittent shedding. When diagnosis is delayed, patients are more likely to have developed more severe symptoms at the time of diagnosis. Delayed diagnosis also allows for increased opportunity to spread infection to others. Prompt diagnosis permits initiation of effective treatment and implementation of infection control measures.

Available Assays

Clinical laboratory detection is routinely performed by microscopic examination of stool specimens using wet mounts and permanent stained slides. Because there is marked fluctuation in the shedding of parasites from day to day, the diagnostic yield of examining a single stool specimen is low (50–60 %); therefore, to improve sensitivity, a total of three stool specimens should be collected every other day or at least on separate days within a period of no more than 10 days [176]. Some parasitic enteropathogens (i.e., *Cyclospora*, *Cystoisospora*, and microsporidia) are difficult to detect in stool and require the use of special stains.

The specificity of microscopic examination is theoretically perfect, but depends on the skill of the microscopist. The sensitivity of microscopic examination also depends largely on the skill of the microscopist and can be low for some parasitic diseases. Concentration techniques are used routinely and improve sensitivity. Since microscopy is very labor-intensive, US FDA-approved immunoassays are available for detecting *Entamoeba histolytica*, *Giardia*, or *Cryptosporidium* antigens in stool, and numerous studies confirm that antigen immunoassays are more sensitive than microscopic examination [177]. Antigen immunoassays have added benefits of being rapid and are technically simple to perform. The sensitivity of microscopy and immunoassay examinations for ova and parasites is low enough that empiric treatment is often given when clinical suspicion is high but tests are negative. Use of X-ray contrast material, laxatives, antacids, or antibiotics (especially tetracyclines and metronidazole), and various other substances can interfere with detection of parasites and delay the diagnosis by as much as several weeks. Due to the nature of the infection, sigmoidoscopy or colonoscopy to obtain duodenal aspirates or biopsy specimens may be necessary for detection of some parasites such as *Cryptosporidium*, microsporidia, or *Giardia*. Even these invasive methods of testing can give false-negative results due the patchy nature of organism distribution in the gastrointestinal tract.

Molecular tools similar to those used for other pathogens are increasingly being used to study parasite polymorphisms and the epidemiology of parasitic diseases. Molecular tests are slowly entering the diagnostic arena and may become more commonly used when US FDA-approved or -cleared tests become available. Current non-molecular diagnostic processes for detection of gastrointestinal parasites can be slow and confusing, while molecular tests have the potential to greatly facilitate diagnosis.

Commercially developed laboratory tests beyond microscopy have been limited to a few well-recognized parasitic pathogens. Individual assays using PCR amplification of parasite DNA sequences extracted from stool or biopsy specimens have been developed for a number of specific

gastrointestinal parasites, but are largely research tools and are not commercially available. Molecular detection of *Cryptosporidium* sp., *Cyclospora cayetanensis*, *Giardia lamblia*, microsporidia, *Entamoeba histolytica*, and *E. dispar* is performed at the Centers for Disease Control and Prevention and may be available at some public health or other reference laboratories.

Multiplex tests are more practical for routine clinical laboratory use and assays for various combinations of parasitic targets have been described in the literature for successful detection of the target organisms in diarrheal stool specimens. The US FDA-cleared xTAG® Gastrointestinal Pathogen Panel (xTAG GPP, Luminex Corporation, Austin, TX) contains primers for the amplification of *Giardia* and *Cryptosporidium* along with other nonparasitic gastrointestinal pathogens. The version of the test marked for diagnostic use in Canada and Europe also detects *Entamoeba histolytica*. The US FDA-cleared BioFire FilmArray™ (bioMerieux, Durham, NC) Gastrointestinal (GI) Panel includes detection of *Cryptosporidium*, *Cyclospora cayetanensis*, *Entamoeba histolytica*, and *Giardia lamblia* in addition to bacterial and viral gastrointestinal pathogens.

Interpretation of Results

In most cases, positive molecular test results in symptomatic patients will correlate with disease. Whereas microscopic examination requires the presence of whole intact parasite for visualization, PCR can only detect the presence of the parasite DNA and cannot distinguish between live, damaged, or dead organisms. Also, the length of time that parasite DNA can be detected after clearance of the organism from the body has not been studied for gastrointestinal parasites, and the possibility that positive results obtained by PCR analysis might be due to lingering parasite DNA may need to be considered, depending on the clinical situation. In addition, asymptomatic carriage is common and must be considered when drawing conclusions about positive results if molecular tests are being used for screening purposes, such as for travelers returning from endemic regions or foreign adopted children.

Laboratory Issues

Stool specimens submitted for microscopic ova and parasite examination are generally placed in preservatives to stabilize parasite morphology and prevent further development of certain helminth eggs and larvae. Preservatives can interfere with PCR-based tests since they act by producing cross-links between nucleic acids and proteins which can obstruct DNA extraction and block PCR amplification. Preservatives also have the potential to cause fragmentation of nucleic acids which could interfere with target amplification. Specimens for molecular tests must be collected without preservatives and kept refrigerated or frozen prior to testing.

Microscopic examination is the most comprehensive method for detection of parasites and has the advantage of allowing detection of any parasite that might be present. Molecular tests are more sensitive but are not developed to the point where microscopy will be completely replaced by PCR. The introduction of real-time PCR assays, especially those that combine several targets into multiplex assays, offers the possibility of using DNA-based detection techniques as a component of a diagnostic approach. However, one of the constraints of multiplex assays is the restriction in the number of parasitic targets that can be detected simultaneously. Additional pathogenic parasites that are not targeted in the molecular assays will still need to be tested for by traditional microscopy or antigen detection methods.

Native genomic DNA and/or whole organisms for culture are available for *Giardia*, *Cryptosporidium* and *Entamoeba histolytica* from ATCC (Manassas, VA) and BEI Resources (Manassas, VA). The NATtrol™ (ZeptoMetrix Corp, Buffalo, NY) verification set contains those same protozoan analytes and also includes *Cyclospora*. Challenges for the molecular detection of *Giardia* and *Cryptosporidium* are available as part of the GIP proficiency survey from the College of American Pathologists.

Future Directions

Since the methods used for pathogen discovery have dramatically improved, emerging gastrointestinal pathogens, such as parechoviruses [178], as well as variants of known pathogens are being recognized at a rapid rate. The rapidly changing catalogue of clinically relevant gastrointestinal pathogens makes development of US FDA-approved/cleared tests difficult.

Clinical laboratories may have difficulty determining when to bring a molecular test into the laboratory. Higher cost is sometimes a deterrent but other considerations are whether a laboratory diagnosis would change patient management, improve outcomes, improve workflow, and/or lower associated healthcare costs. Sometimes molecular tests are so far superior to existing diagnostic tests that the issue is not whether a clinical laboratory can afford to offer a molecular test, but rather whether the laboratory can afford not to when the total cost of healthcare is considered.

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