Expert Review of Molecular Diagnostics

ISSN: 1473-7159 (Print) 1744-8352 (Online) Journal homepage: http://www.tandfonline.com/loi/iero20

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To cite this article: Yuliya Zboromyrska & Jordi Vila (2016) Advanced PCR-based molecular diagnosis of gastrointestinal infections: challenges and opportunities, Expert Review of Molecular Diagnostics, 16:6, 631-640, DOI: 10.1586/14737159.2016.1167599

To link to this article: http://dx.doi.org/10.1586/14737159.2016.1167599

Accepted author version posted online: 17 Mar 2016.
Published online: 01 Apr 2016.

EXPERT REVIEW OF MOLECULAR DIAGNOSTICS, 2016
VOL. 16, NO. 6, 631–640
http://dx.doi.org/10.1586/14737159.2016.1167599

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REVIEW

Advanced PCR-based molecular diagnosis of gastrointestinal infections: challenges and opportunities

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ABSTRACT

Acute infections of the gastrointestinal tract are among the most common infectious diseases. The etiological agents of gastroenteritis may be bacteria, viruses or protozoa. Identification of the etiological agents of acute diarrhea is important for the treatment and management of diarrheal diseases. Conventional stool culture for bacteria shows a low sensitivity and requires more than 24 hours. In addition, other approaches to detect viruses and protozoa mainly involve antigen detection, but this is not available for all enteropathogens, and microscopic observation requires training and is of low sensitivity. In this review, the authors describe currently available molecular methods to detect different enteropathogens and analyze the main advantages and disadvantages of these methods for laboratory diagnosis of gastroenteritis.

ARTICLE HISTORY
Received 7 January 2016
Accepted 15 March 2016
Published online
1 April 2016

KEYWORDS
Gastroenteritis; diarrhea; enteropathogens; bacteria; viruses; protozoa; DNA-based tools; culture; antigen test; microscopic observation

Introduction

Acute gastrointestinal infections are among the most common infectious diseases worldwide, being surpassed only by respiratory tract infections. Although electrolyte imbalance caused by gastrointestinal infections may often cause a slight setback in healthy adults, it can produce dehydration in very sick people, children, and the elderly. Globally, gastrointestinal infections remain a major cause of morbidity and mortality among infants and children [1,2]. Diarrhea epidemics in infants, children, and adults are usually caused by microorganisms present in the water, food, or hospital environment. Infections can also be transmitted from person to person by direct contact or through fomites [3,4]. The etiological agents of gastroenteritis may be bacteria, viruses, or protozoa, with the prevalence of the different microorganisms depending on several aspects such as (1) community versus nosocomial acquisition, (2) children versus adults, (3) acquisition in developing versus developed countries, (4) patients who have traveled abroad (traveler's diarrhea) and those who have not, and (5) immunocompetent versus immunocompromised patients. [5–13].

Conventional laboratory techniques

Detection and identification of the etiological agents of acute diarrhea are important for not only the treatment of individual patients but also for the management of diarrheal diseases from the public health point of view. Conventional culture remains the gold standard for bacterial enteropathogens, even though stool culture has a relatively low sensitivity and is laborious [14]. Ova and parasite examination is ordered to determine the possible presence of parasites in the digestive tract. Microscopy is used for visualization, but it has a low sensitivity and is time consuming and requires specific training to differentiate ova and parasites [15]. In addition to ova and parasite examination antigen tests may also be used to detect *Giardia lamblia, Cryptosporidium* spp., or *Entamoeba histolytica*. However, antigen tests can only detect a few specific parasites and therefore cannot replace ova and parasite examination which is able to detect a wider variety of parasites. Antigen tests have also been used to detect viruses causing gastroenteritis such as rotavirus and adenovirus; however, these tests are not available for all enteropathogens and show a variable sensitivity.

In the last decade, some tools involving mainly polymerase chain reaction (PCR)-based techniques have been developed to detect specific microorganisms or a panel of different enteropathogens directly from stool samples [16].

Molecular diagnostic tools

Single pathogen detection assays

Several molecular assays are available for the detection of a single gastrointestinal pathogen, making review of all these assays difficult. Nevertheless, taking into account the clinical and epidemiological relevance, we will mainly focus on the detection of toxigenic *Clostridium difficile*, as the leading cause of hospital-acquired infection, and noroviruses, which has been increasingly identified as the cause of community-acquired infections as well as of hospital outbreaks [17–22].

C. difficile

Several molecular assays have been designed for the diagnosis of infection by *C. difficile*. Thus, Jensen *et al*. compared four

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molecular tests with the toxigenic culture [23]. These tests included illumigene® *C. difficile* (Meridian Bioscience, Milan, Italy) (targeting *tcdA* and *tcdB*), PCRFast® *C. difficile A/B* (ifp, Institut für Produktqualität, GmbH, Berlin, Germany) (targeting *tcdA* and *tcdB*), GeneXpert® *C. difficile*/Epi (Cepheid, Sunnyvale, CA, USA) (*tcdA, tcdB*, Δ117*tcdC,* and *cdt*), and an in-house real-time polymerase chain reaction (rt-PCR) (*tcdA, tcdB,* and *cdtA* followed, if positive, by the detection of 3 *tcdC* alleles). Although the overall agreement of the four molecular tests was similar, the sensitivity of PCRFast was significantly lower (76.3%) compared with those of GeneXpert (100%), illumigene (94.7%), and the in-house PCR (97.4%). Importantly, PCRFast was found to be more sensitive to inhibitors present in stool samples, with 40 (13.4%) of initially inhibited samples detected among the 299 processed. Therefore, the inhibited samples required retesting after dilution, thereby increasing the turnaround time as well as costs. On the other hand, GeneXpert (two inhibited samples), illumigene (eight inhibited samples), and in-house rt-PCR (no inhibition) were found to be more robust when performed with fecal samples. In this study, molecular assays also showed greater sensitivity than a conventional toxigenic culture. To improve the diagnosis, the authors performed an expanded toxigenic culture that mainly included prolonged incubation and re-culture of samples and were able to detect additional positive samples. Nevertheless, 16 stool samples positive by at least 2 molecular tests remained negative by culture. Another advantage offered by molecular tests is the detection of hypervirulent ribotypes 027 and 066/078 of *C. difficile* (GeneXpert® *C. difficile*/Epi and in-house PCR), which may have an impact on patient management and the rapid application of epidemiological control measures. Finally, it is important to highlight that GeneXpert and illumigene are integrated systems that include extraction and amplification/detection steps with an important reduction in turnaround time, making these assays easy to perform.

In another study, highlighting the high sensitivity of GeneXpert, Berry *et al*. [24] studied the concordance between GeneXpert, the cell culture cytotoxin neutralization assay (CCNA), and glutamate dehydrogenase (GDH), this later test normally yields a specificity of 80–100%. They found 89.9% (886/985) of the samples to be concordant. GeneXpert showed a higher detection rate (11.7%) than CCNA (6%). The detection rate of GDH alone was the highest (13.8%) as this test does not differentiate toxigenic from non-toxigenic strains. However, when GDH-positive samples were tested for *C. difficile* toxins A and B (enzyme immunoassay) and samples were considered as negative if no toxins were detected, the rate of positivity decreased and 59.7% of patients with clinical *C. difficile* infection might have been missed. On the other hand, the results of the three assays were compared with the clinical diagnosis as the reference. The sensitivity of GeneXpert, CCNA, and GDH alone was 99.1%, 51%, and 83.8%, respectively. Interestingly, of 59 GeneXpert-positive and CCNA-negative results, 54 were considered as true positives based on the clinical diagnosis.

Despite the high sensitivity of molecular tests for *C. difficile* detection, the cost of the application of these tests in all the stool samples received by a clinical microbiology laboratory is high. Therefore, the use of two-step approaches, including the first screening with GDH alone or in combination with A/B toxin detection followed by a PCR-based test, has been widely studied [25]. Culbreath *et al*. showed that a protocol including GeneXpert as the confirmation test for GDH-positive and toxin-negative results had a higher sensitivity compared with the cytotoxin neutralization assay, reducing the turnaround time and saving costs [26].

Nevertheless, several studies have described the lack of specificity of molecular assays for the diagnosis of infection by *C. difficile* taking into account that it is a toxin-mediated disease. Recently, Polage *et al*. performed a prospective observational cohort study including 1416 hospitalized patients to determine the need for treatment of patients with positive PCR and negative toxin immunoassay results [27]. Stool samples were tested with a *C. difficile* toxin immunoassay and the results were reported to clinicians; in addition, one or two molecular tests (GeneXpert and illumigene) were also performed but the results were not reported. The patients were divided into three groups: toxin-positive and PCR-positive (Tox +/PCR+), toxin-negative (Tox–)/PCR+, and Tox–/PCR-negative (PCR–). Interestingly, 55.3% of patients with PCR+ results were negative by the toxin immunoassay. The Tox+/PCR+ group showed an increased *C. difficile* load in feces, a higher toxin concentration, longer duration of diarrhea, more *C. difficile* infection-related complications, and mortality, compared with the Tox–/PCR+ group. Moreover, no significant differences were observed in patient outcome between the Tox –/PCR+ and Tox–/PCR– groups, despite 59.3% of patients in the Tox–/PCR+ group not receiving any or only partial treatment (27.8%) for *C. difficile* within the first 14 days. Based on the study results, the authors suggested that patients with PCR+ and toxin detection-negative results should not be treated as they were probably colonized with *C. difficile* – a common clinical condition among hospitalized patients [28,29]. Similar findings were reported by Planche *et al*., who demonstrated that PCR+ (GeneXpert) and cell cytotoxin assay-negative results did not predict a poorer outcome or prolonged hospital stay [30].

Noroviruses

Noroviruses are major enteropathogens worldwide, producing outbreaks as well as sporadic cases of diarrhea. A recent study reported that the number of cases during noroviruses outbreaks in the hospital setting was significantly lower (P = 0.028), if microbiological test results for noroviruses were available before the onset of the outbreak [21]. rt-PCR is the method most frequently used for the detection of noroviruses. Hyun *et al*. compared two commercial rt-PCRs, the AccuPowerNorovirus rt-PCR Kit (Bioneer Co., Daejeon, South Korea) and RIDAGENE Norovirus (R-Biopharm, Darmstadt, Germany), for the detection of noroviruses GI/GII [31]. A total of 281 stool samples were processed, including 109 positive and 172 negative samples according to a noroviruses enzyme-linked immunosorbent assay (ELISA). Interestingly, the overall agreement was higher between the two PCR tests than between ELISA and each of the PCR assays. The positive percent agreement between AccuPower and RIDAGENE was 99.0%, whereas the negative percent agreement was 95.1%. A recent evaluation of the Xpert Norovirus

assay for the detection and differentiation of noroviruses GI and GII carried out in a multicenter study, demonstrated a positive percent agreement of 98.3% for GI and 99.4% for GII, and a negative percent agreement of 98.1% for GI and 98.2% for GII [32]. Despite most gastrointestinal infections being caused by the GI and GII noroviruses, genogroup IV can also produce an acute gastroenteritis in humans [33,34]. For this reason, the detection of genogroup IV has recently been included in some PCR-based assays [35]. Farkas *et al*. developed a single-tube multiplex PCR assay for the detection and quantification of GI, GII, and GIV noroviruses which demonstrated a sensitivity and specificity equal to the monoplex tests [36].

Multiple pathogen detection assays

Considering the wide range of pathogens that can cause diarrhea and the similarity of the clinical manifestations of disease produced by different pathogens, the multiplex molecular assays seem to be an optimal tool for rapid diagnosis. Despite the development of numerous in-house tests, demonstrating a high sensitivity and specificity, the commercial molecular panels offer several advantages and facilitate the comparison of results between laboratories. Several commercial molecular panels are available (Table 1), one of the most evaluated being the xTAG® gastrointestinal pathogen panel (GPP) (Luminex Molecular Diagnostics, Toronto, Canada) that allows the simultaneous detection of 15 pathogens, including bacteria (*Salmonella* spp., *Campylobacter* spp., *Shigella* spp., *C. difficile* toxin A/B, enterotoxigenic *Escherichia coli* (ETEC) LT/ST, *E. coli* O157, shiga-like toxin-producing *E. coli* (STEC), *Yersinia enterocolitica, Vibrio cholerae*), viruses (adenovirus 40/41, rotavirus A, and norovirus GI/GII) and parasites (*G. lamblia, E. histolytica,* and *Cryptosporidium* spp.). This method has a turnaround time of about 5 h and includes separate nucleic acid extraction, amplification, and hybridization steps. In 2013, Navidad *et al*. evaluated the xTAG panel for the detection of enteropathogens of clinical and public health importance [14]. The study was performed with 48 isolates to spike stools and 254 clinical stool samples from outbreak and sporadic cases of gastroenteritis. GPP showed 100% sensitivity and specificity using 48 spiked stool samples. Regarding 254 clinical samples, the concordance with conventional testing was ≥90% for all the pathogens identified. GPP worked well with raw stool samples as well as samples in Cary–Blair transport media. The main limitation observed in this study was the detection of 29 samples positive for *E. histolytica*, 5 of which were confirmed by rt-PCR and 24 remained unresolved by either rt-PCR or microscopy.

In the same year, Claas *et al*. compared GPP with conventional diagnostic procedure. A total of 901 stool samples from 4 clinical sites were processed [38]. The sensitivity of GPP for parasites and viruses ranged between 91.3% and 100%, with the exception of adenovirus for which the sensitivity was only 20% compared with rt-PCR. Nevertheless, the authors suggested that most of adenoviruses detected by rt-PCR and not by GPP were actually types other than 40/41. This hypothesis was supported by the sequencing results of a subset of discordant samples. Regarding bacteria, the sensitivity of the test for *Salmonella* (82.7%), *Campylobacter* (97.4%), *Shigella* (100%), STEC (100%), *C. difficile* (96.7%), and *E. coli* O157 (93.7%) was determined. Microbiological testing was not requested by clinicians for a high number of pathogens detected by GPP (65% of positive samples). Interestingly, GPP detected multiple pathogens in 9.5% of stool samples. This finding was also reported in a previous study in which the performance of GPP was evaluated with samples from patients with suspected traveler's diarrhea, detecting multiple pathogens in 28.6% of positive samples [8]. In this study, the rate of positivity by GPP also increased with the detection of an additional 60 pathogens which were not detected by conventional techniques or not requested by clinicians. Lalani *et al*. demonstrated that multiplex PCR using xTAG analyte-specific reagents for the detection of enteropathogens could be effective not only with direct fecal samples but also with stool smears on a filter paper matrix (Whatman™ FTA Elute cards [GE Healthcare Bio-Sciences Pittsburgh, PA]) [53].

The FilmArray GI panel (FilmArray) (BioFire, Inc., Salt Lake City, UT) is another multiplex platform covering the majority of enteropathogens. Initially, this panel allows the detection of 23 targets, including 14 bacteria (*Aeromonas* spp., *Campylobacter, C. difficile* toxin A/B, *Plesiomonas shigelloides, Salmonella, Y. enterocolitica, Vibrio* spp., enteroaggregative *E. coli* [EAEC], enteropathogenic *E. coli* [EPEC], ETEC, STEC, *E. coli* O157, and enteroinvasive *E. coli* [EIEC]/*Shigella*), 5 viruses (adenovirus 40/41, norovirus GI/GII, rotavirus A, sapovirus, and astrovirus), and 4 parasites (*Cryptosporidium, Cyclospora cayetanensis, E. histolytica,* and *G. lamblia*). Interestingly, *Aeromonas* was the only target under investigation-use only included in the panel. Currently, FilmArray no longer yields results for *Aeromonas* spp. FilmArray is an integrated platform which requires only 5 min of hands-on time and has a turnaround time of 1 h. In 2014, Khare *et al*. compared the performance of FilmArray and GPP with conventional microbiological testing in 230 prospective stool samples and 270 characterized stool samples in Cary–Blair medium [39]. FilmArray and GPP increased the rate of positivity among prospective stool samples to 33% and 30%, respectively, compared with 8.3% of positive samples detected by conventional methods. However, after confirmation testing of discordant results the rate of positivity was 28.3% for FilmArray and 20% for GPP. Regarding the performance of both tests with 270 previously characterized stool samples (27 negative and 243 positive), the sensitivity of FilmArray ranged between 90% and 100% for all targets, except for *Aeromonas* (23.8%) with 16 false negative results and *E. histolytica* (0%) with one false negative. GPP showed a lower sensitivity for several pathogens including *Salmonella* (83.3%), EIEC/*Shigella* (81.8%), adenovirus 40/41 (80%), *Campylobacter* (79.3%), and especially *Y. enterocolitica* (48.1%). Similar to other studies, both molecular panels detected a high percentage of mixed infections among processed samples (27% by FilmArray and 14.1% by GPP). Finally, the specificity of the two assays was high with the exception of noroviruses detected by GPP. Interestingly, retesting of these discordant samples with a new kit showed a negative result in 31 out of 32 initially noroviruses positive samples. Recently, Spina *et al*. published the results of the

Table 1. Comparison of the different commercial multiplex molecular assays.

Assay	The targets included	Technology	Integrated system	Hands-on time (min)	Turnaround time (h)	Number of samples per run	Refs.
GPP (Luminex, Molecular Diagnostics, Toronto, ON, Canada)	Bacteria: *Salmonella, Campylobacter, Shigella, Clostridium difficile* toxin A/B, ETEC, *Escherichia coli* O157, STEC, *Yersinia enterocolitica*, and *Vibrio cholerae* Viruses: adenovirus 40/41, rotavirus A, and noroviruses GI/GII Parasites: *Giardia lamblia, Entamoeba histolytica*, and *Cryptosporidium*	Multiplex rt-PCR and hybridization with detection by fluorescent-labeled bead array	No	≈45	≈5	Up to 96	[8,37–43]
FilmArray (Biofire Diagnostics, Salt Lake City, UT, USA)	Bacteria: *Campylobacter, C. difficile* toxin A/B, *Plesiomonas shigelloides, Salmonella, Y. enterocolitica, Vibrio*, EAEC, EPEC, ETEC, STEC, *E. coli* O157, and *Shigella*/EIEC Viruses: adenovirus 40/41, noroviruses GI/GII, rotavirus A, sapovirus, and astrovirus Parasites: *Cryptosporidium, Cyclospora cayetanensis, E. histolytica*, and *G. lamblia*	Nested PCR, multiplex rt-PCR with endpoint melt curve analysis	Yes	≈2–5	≈1	1	[39,44–46]
Seeplex (Seegene, Seoul, Korea)	Bacteria: *Salmonella, Shigella, Vibrio, C. difficile* toxin B, *Campylobacter, Clostridium perfringens* toxin, *Y. enterocolitica, Aeromonas, E. coli* O157, and VTEC Viruses: rotavirus A, adenovirus 40/41, astrovirus, and noroviruses GI/GII	Multiplex rt-PCR with detection by auto-capillary electrophoresis device	No	≈45	≈9–10	Up to 96	[40,47,48]
TaqMan Array Card platform (Life Technologies, Foster City, CA, USA)	Bacteria: ETEC, EPEC, EAEC, STEC, *Shigella*/EIEC, *Salmonella, Campylobacter, V. cholerae,* and *C. difficile* Viruses: rotavirus, norovirus GII, adenovirus, astrovirus, and sapovirus Parasites: *Cryptosporidium, G. lamblia, E. histolytica, Ascaris lumbricoides,* and *Trichuris trichiura*	A combination of several singleplex rt-PCR on one 384-well card with detection by hydrolysis probes labeled with quenched fluorophores	No	≈45	≈3	Up to 8	[49]
BD MAX™ Enteric Bacterial Panel (Becton Dickinson GmbH, Heidelberg, Germany)	*Salmonella, Campylobacter, Shigella*/EIEC, and STEC	Multiplex rt-PCR with detection by hydrolysis probes labeled with quenched fluorophores	Yes	≈20	≈3	1	[50,51]
RIDA®GENE (R-Biopharm AG, Darmstadt, Germany)	Bacterial Stool Panel: *Salmonella, Campylobacter, Shigella*/EIEC, and STEC EHEC/EPEC panel Other#	Multiplex RT and rt-PCR with detection by hydrolysis probes labeled with quenched fluorophores	No	≈45	≈3	Several*	[50]
FTD® Gastroenteritis Panel (Fast Track Diagnostics, Junglinster, Luxembourg)	FTD Bacterial gastroenteritis panel: *Salmonella, Campylobacter, Shigella*/EIEC, *C. difficile*, STEC, and *Y. enterocolitica* FTD Viral gastroenteritis panel: adenovirus, noroviruses GI/GII, rotavirus A, sapovirus, and astrovirus FTD Stool parasites panel: *G. lamblia, E. histolytica,* and *Cryptosporidium*	Multiplex RT and rt-PCR with detection by hydrolysis probes labeled with quenched fluorophores	No	≈45	≈3	Several*	[50]
proGastro SSCS (Gen-Probe Prodesse, San Diego, CA)	*Salmonella, Campylobacter, Shigella* and, STEC	Multiplex rt-PCR with detection by hydrolysis probes labeled with quenched fluorophores	No	≈45	≈3–4	Several*	[52]

rt-PCR: Real-time polymerase chain reaction; RT: reverse transcription; ETEC: enterotoxigenic *Escherichia coli*; EPEC: enteropathogenic *E. coli*; EAEC: enteroaggregative *E. coli*; STEC: shiga-toxigenic *E. coli;* EIEC: enteroinvasive *E. coli;* EHEC: enterohemorrhagic *E. coli*; VTEC: verotoxin-producing *E. coli*.
*Depending on thermocycler used (RIDA®GENE: LightCycler® (Roche), SmartCycler® (Cepheid), m2000rt (Abbott), Rotor-Gene Q (Qiagen), ABI 7500, Mx3005P, and CFX96™; FTD: LightCycler® or SmartCycler®; proGastro SSCS: SmartCycler®).
#Several RIDA®GENE panels with different combination of enteropathogens are available.

first European, multicenter, cross-sectional quarterly point-prevalence study evaluating FilmArray for the diagnosis of community-acquired gastroenteritis [44]. Clinical microbiology laboratories from 10 European countries participated in the study. On four separate days in 2014 stool samples were collected for conventional on-site testing and a 500 µl-aliquot in Cary–Blair medium was sent to the central study laboratory in Vienna, Austria. A total of 709 stool samples were analyzed with the overall positivity rate being 54.2%. The highest positivity rate was observed in samples from

Romania (74%). Mixed infections were identified in 16.4% (116/709) of samples with the most common coinfection being of *Campylobacter* with EPEC. These mixed infections may represent sometime a problem for the interpretation of the results. Regarding entero-pathogens detected by FilmArray, the six most prevalent were EPEC, *Campylobacter, C. difficile*, EAEC, noroviruses, and ETEC, with some differences depending on the country. On analyzing conventional testing results, only 18.1% of the samples were positive, with *Campylobacter* being the most frequent pathogen, followed by *Salmonella, C. difficile*, rotaviruses, and noroviruses. No country performed routine testing for EAEC and only three did so for EPEC. This study again confirmed the high sensitivity of molecular panels for enteropathogen detection and the suboptimal efficiency of the current conventional diagnostic procedures. Moreover, the need to test for some pathogens not included in routine testing, such as EPEC and EAEC, was also demonstrated.

Onori *et al*. compared another multiplex PCR-based assay (Seeplex® Diarrhea ACE Detection; Seegene, Seoul, Korea) with the routine procedure for the diagnosis of infectious gastroenteritis in children. This test allows the detection of 10 bacteria and toxins (*Salmonella* spp., *Shigella* spp., *Vibrio* spp., *C. difficile* toxin B, *Campylobacter* spp., *Clostridium perfringens* toxin, *Y. enterocolitica, Aeromonas* spp., *E. coli* O157:H7, and verotoxin-producing *E. coli*) and 4 viruses (rotavirus A, adenovirus 40/41, astrovirus, and norovirus GI/GII) [47]. This system also required a separate sample preparation and extraction step, and PCR was performed in three separate reaction tubes. The ScreenTape system was used for the detection. Parasitic infection was not evaluated in this study. All the discordant results were confirmed by in-house PCR performed with the extracted stool samples, and confirmed results were included to calculate the sensitivity and specificity of the conventional and Seeplex methods. A total of 245 liquid stools samples were processed. Conventional testing detected 17.6% samples positive for bacterial pathogens, 43.2% for viruses, and 5.7% bacterial–viral coinfections with an overall rate of positivity of 66.5%. Seeplex detected 14.7% samples positive for bacteria, 49.4% for viruses, and 12.2% of coinfected samples with an overall rate of positivity of 76.3%. Seeplex showed a higher sensitivity for almost all the pathogens analyzed except for *Salmonella* and toxigenic *C. difficile* that could be explained by the use of enrichment broth for *Salmonella* and the performance of rt-PCR (GeneXpert) with isolated colonies of *C. difficile* in the routine procedure. In a previous study, Coupland *et al*. reported a lower sensitivity of Seeplex compared with conventional testing for the detection of *Salmonella* and *C. difficile* [48].

Liu *et al*. developed a multiplex assay based on a TaqMan Array Card platform (TAC) (Life Technologies, Foster City, CA, USA), which allows the detection of 19 pathogens, including 9 bacteria (*Campylobacter* spp., *Salmonella* spp., *C. difficile, V. cholerae*, EAEC, EPEC, ETEC, STEC, and *Shigella*/EIEC), 5 viruses (adenovirus, astrovirus, norovirus GII, rotavirus, and sapovirus), 3 protozoa (*Cryptosporidium* spp., *G. lamblia,* and *E. histolytica*), and helminths (*Ascaris lumbricoides* and *Trichuris trichiura*) [49]. TAC is a 384-well microfluidic card with a panel of dried singleplex assay mixtures. The TAC platform offers the possibility to create different pathogen combinations if PCR conditions needed to amplify each target are similar. Nevertheless, nucleic acid is extracted separately. This assay was validated with 109 stool samples from Tanzania and Bangladesh showing a sensitivity of 85% and specificity of 77% compared with conventional testing. In the same study, the results of TAC were also compared with the PCR-Luminex panels described previously, showing a sensitivity of 98% and specificity of 96%.

Biswas *et al*. compared three molecular panels for the detection of *Salmonella* spp., *Shigella* spp., *Campylobacter* spp., and STEC: RIDA®GENE Bacterial Stool and enterohemorrhagic *E. coli*/EPEC Panels (RIDA) (R-Biopharm AG, Darmstadt, Germany); FTD® Bacterial Gastroenteritis Panel (FTD) (Fast Track Diagnostics, Junglinster, Luxembourg); and BD MAX™ Enteric Bacterial Panel (BD MAX) (Becton Dickinson GmbH, Heidelberg, Germany) [50]. Both community and hospitalized patients were included in the study with a total of 434 stool samples being analyzed. The three panels demonstrated a greater sensitivity than culture for the detection of *Campylobacter* spp. and *Shigella* spp., while the sensitivity of RIDA and FTD was low for *Salmonella* spp. (25% and 50%, respectively). As RIDA and FTD required separate DNA extraction, the hands-on time of these two panels was longer (50 min) compared with BD MAX (20 min). BD MAX is an integrated system incorporating sample preparation, extraction, amplification, and detection. In 2015, Knabl *et al*. compared BD MAX with a conventional method for the detection of the same four bacterial pathogens [51]. Of the 971 stool samples included in the study, invalid results were obtained in 78 due to inhibition or technical errors and retesting of samples still available was required. The positive percent agreement of BD MAX and conventional methods for *Campylobacter* spp., *Salmonella* spp., *Shigella* spp., and STEC was 0.97, 0.75, 1.00, and 0.88, respectively. BD MAX increased the rate of positivity from 5.26% to 8.06% among processed samples by the detection of an additional 13 *Campylobacter*, 8 *Salmonella*, 3 *Shigella,* and 5 STEC. ProGastro SSCS is another commercial rt-PCR-based test which has been evaluated for the detection of these four bacterial pathogens causing infectious gastroenteritis. This assay requires a separate extraction step and includes two PCR master mixtures: one for the detection of *Salmonella, Shigella,* and *Campylobacter* and another for STEC (*stx1* and *stx2* genes). Amplification is carried out in a SmartCycler and the turnaround time is 3–4 h. Buchan *et al*. compared the ProGastro panel with conventional methods including culture and enzyme immunoassay [52]. During the study 1139 prospective stool samples were collected from four clinical centers and processed. The overall sensitivity of ProGastro was 98.5%, while the specificity ranged between 98.9% and 99.4%, depending on the target detected. Nevertheless, when bidirectional sequencing was performed with the discrepant samples (mainly false positive ProGastro results), the sensitivity of the assay was 100% for all four pathogens and the specificity increased to 99.4–100%. Despite the high sensitivity demonstrated by RIDA, FTD, BD MAX, and ProGastro, the main limitation of these assays is that only a limited number of bacterial pathogens can be detected on comparison with other assays such as GPP, FilmArray GI, or

Seeplex, which cover a wider range of bacterial pathogens causing gastroenteritis. This is also the case of the EntericBio real-time Gastro Panel I (Serosep, Limerick, Ireland), which allows the simultaneous detection of *Campylobacter* spp., STEC, *Shigella* spp., and *Salmonella* spp. [54]. Nevertheless, it is important to highlight that separate panels are commercially available or under development for the detection of viruses and parasites causing gastroenteritis by the RIDA, FTD, and BD MAX assays.

Coste *et al*. demonstrated the utility of multiplex molecular panels for the diagnosis of infectious gastroenteritis in kidney transplant recipients. Seven commercial PCR-based assays were tested with samples collected from patients (n = 54) at the time of the severe diarrhea episode and 6 months after resolution, renal transplant recipients (n = 30) with no history of diarrhea, and asymptomatic immunocompetent patients (n = 30). The authors showed that molecular assays significantly increased the detection rates comparing with conventional methods and also improved the detection of coinfections [40].

Several other multiple-pathogen detection assays are commercially available including Faecal Pathogens B (AusDiagnostics, Beaconsfield NSW, Australia) [55], the multiplex PCR tandem-based assay, which allows the detection of 6 bacteria, 5 viruses, and 3 parasites; Allplex (Seegene, Seoul, Korea) [56], multiplex rt-PCR assay, which includes 4 separate panels for the detection of 13 bacteria, 5 viruses, and 6 parasites; the Verigene Enteric Pathogens Test (Nanosphere, Inc., Northbrook, IL) [57], an integrated platform, which allows the detection of 6 bacteria and 2 viruses in 2 h with 5 min of hands-on time; CLART EnteroBac (Genomica, Madrid, Spain) [58], a multiplex PCR with array detection of 7 bacteria in approximately 5 h; among others. Most of these molecular panels have only been evaluated by the manufacturers. Thus, further studies are needed in clinical laboratory settings to assess their accuracy for the diagnosis of infectious gastroenteritis.

How useful are multiplex molecular panels for patients and hospitals?

Although the utility of an early detection of *C. difficile* and noroviruses in the hospital setting has been widely demonstrated [19,59,60], few studies published have evaluated the clinical and epidemiological utility of multiplex gastrointestinal assays for the diagnosis of infectious gastroenteritis. Halligan *et al*. evaluated several parameters related to the utility of GPP testing compared to a conventional procedure over an 8-month period in an 1100-bed hospital [41]. A total of 2187 stool samples were processed by both methods, with 55% of samples from hospital-acquired and 45% from community-acquired diarrhea cases. Apart from the higher sensitivity of GPP for the detection of some pathogens and mixed infections, discussed above, this study highlighted the utility of multiplex assays to improve the use of isolation facilities in hospitals with a limited number of isolation rooms, as 60% of community-acquired and 42% of hospital-acquired cases were removed from isolation following a negative GPP result. On the other hand, 57 patients were isolated based on GPP-positive results. Therefore, a clear benefit was demonstrated in terms of isolation stay saving. Regarding costs, the authors compared the laboratory costs of GPP and conventional testing, with the former clearly presenting higher costs compared to those of the conventional procedure. Nevertheless, as pointed by the authors, a cost-effectiveness analysis should be performed in order to calculate the global costs of gastroenteritis, based on patients outcome, isolation stay saving, shorter emergency unit stay, and avoidance of hospital admission in some cases, among others.

Addressing the need for an economic evaluation of the implementation of multiplex assays in laboratories, Goldenberg *et al*. performed a cost–benefit analysis of GPP in hospitalized patients [42]. The analysis included laboratory and patient isolation costs. The overall costs of GPP testing were higher (£56,243) than those of conventional methods (£33,960). Nevertheless, the total isolation time was 755 days less with GPP testing, generating costs savings of £66,765 and resulting in a global cost saving of £44,482.

Rand *et al*. evaluated the usefulness of FilmArray to detect unsuspected agents of acute gastroenteritis among inpatients [45]. For this, 158 stool samples negative for *C. difficile* and/or rotavirus were processed by FilmArray. In 22.2% of the samples, at least one unsuspected pathogen was detected, mainly noroviruses, rotaviruses, and EPEC. In 60% of patients with unsuspected pathogens, no enteropathogens were detected. The authors also reported two clusters of rotavirus infection as a result of possible nosocomial transmission. FilmArray was also evaluated in another study performed in a population of children and young adults with acute gastroenteritis to compare the diagnostic yield of microbiological testing ordered by clinicians [46]. An important number of additional pathogens were detected using FilmArray in 29% of samples in which only *C. difficile* testing was requested, in 57% of samples in which *C. difficile* and other tests were requested, and in 63% of samples in which *C. difficile* testing was not requested. Thus, the multiplex panels could be useful for effective hospital isolation of patients with diarrhea caused by pathogens not included in conventional testing.

In the study of Pankhurst *et al.,* the main potential benefits for infection control of acute gastroenteritis identified were improvement of the decision for isolation and de-isolation of patients, improvement of patient outcomes, and early detection of outbreaks [14].

Limitations of molecular diagnostic assays

Among the characteristics of the multiplex molecular platforms discussed above, GPP covers more than 80% of the pathogens involved in infectious gastroenteritis and provides results in a single working day. Up to 96 samples can be simultaneously processed. This assay also showed a high sensitivity for most pathogens included in the panel and efficient detection of coinfections. However, this method includes several manual steps during sample preparation, separate nucleic acid extraction, amplification, hybridization, and finally detection with a hands-on time about 45 min. Seeplex has the same limitations. This increases the risk of contamination and makes these panels more labor intensive in comparison with

integrated systems such as FilmArray or BD MAX. FilmArray is an integrated system that offers the advantage of being a rapid and easy-to-use method. In addition, this panel includes a wider range of target genes (22 vs. 15 of GPP), but can run only one sample at a time. This panel also has a higher cost per sample processed [39]. On the other hand, the BD MAX Enteric Bacterial Panel allows the simultaneous analysis of 24 samples, but includes the detection of only 4 bacterial pathogens and may be used in combination with viral and parasitic detection panels to cover the majority of enteropathogens causing infectious gastroenteritis.

Almost all the studies evaluating multiplex molecular panels showed a subset of unconfirmed results [8,37,39]. These could be explained by nonspecific amplification, representing, therefore, false positive results, by the use of less sensitive methods for confirmation testing or the lack of remaining sample for additional confirmation testing. In any case, the clinical significance of reporting these results should be studied. Although molecular panels demonstrated a higher sensitivity than the culture method for the detection of most bacterial enteropathogens, the detection of *Salmonella* seems to be challenging for several molecular assays [43,47,50,61]. The differences in the detection of this pathogen may be due to the different genes used for the detection [50], non-efficient extraction methods [43], or to the low bacterial load in the sample, which could be increased by an enrichment step included in the routine procedure [47]. In addition, false negative results for RNA viruses, with a high mutation rate, may be due to mutations which affect primer binding sites [62].

Most papers presenting a case-control study on the etiology of diarrhea in different settings show the detection of enteropathogens in asymptomatic patients. In the study published by Frickmann *et al*., 410 stool samples from healthy Madagascan school children were tested with multiplex rt-PCR for the detection of enteroinvasive bacteria, enteric protozoa, and helminths [63]. Interestingly, only 26.1% of samples were negative. This finding highlights the problem of the interpretation of positive results obtained by molecular diagnostics methods, especially in areas of high endemicity. Nevertheless, a similar percentage of positivity was also reported in samples from asymptomatic children in the Netherlands [64]. In this study, 95.4% of stool samples analyzed by several multiplex rt-PCR for the detection of enteropathogens were collected from asymptomatic children with the overall rate of positivity being 78%. PCR-based stool pathogen quantification may help distinguish clinically significant infections [65]. However, this is another important limitation of these methods since although quantitative detection is implicit to rt-PCR, not all current multiplex panels allow the quantitation of the different enteropathogens detected. Viral load thresholds to distinguish between symptomatic and asymptomatic infections have been estimated for some enteropathogens, not always with clear-cut conclusions [66–68].

Another important limitation of multiplex panels is that the combination of pathogens to be detected is frequently not optimal and cannot be changed by the user. This point was discussed in depth in a recently published report by Schreckenberger and McAdam [69]. Therefore, there is a need for a 'user-open' platform that would allow the combination of different pathogens detection assays in a multiplex reaction depending on the clinical and epidemiological characteristics of the patients and local epidemiology. The ARIES® system developed by Luminex may be an example of this type of platform [70]. This integrated system carries out extraction, purification, amplification, and detection steps in a single cassette and allows users to perform different singleplex or multiplex laboratory-developed rt-PCR-based assays by loading the corresponding PCR reagents in a cassette. In addition, all types of samples are admitted. This interesting platform seems to be a promising future approach in the field of molecular diagnostics although it should be further evaluated in the setting of microbiology laboratories.

Expert commentary

The main advantages of the molecular tools to diagnose gastroenteritis (Table 2) are (1) the diagnostic yield is increased mainly for some specific microorganisms such as *Shigella, Campylobacter*, toxigenic *C. difficile, Giardia*, etc.; (2) workflow is improved since conventional methods to isolate bacteria may take more than 24 h and with these molecular approaches the turnaround time is between 1 and 5 h depending on the method used; (3) the hands-on-time is also reduced since bacteria, viruses, and parasites can be detected in one panel, thereby considerably reducing the time needed by conventional methods involving cultures, preparation of samples to visualize the protozoa by microscopy, or the tools used to detect viruses such as immunochromatography; and (4) they may have an impact on infection control and costs. However, several of these molecular methods may have some disadvantages such as (1) some microorganisms can be shed in feces for several weeks following infection and PCR detects low levels of the microorganism, which may not be clinically relevant [65,71]; (2) the detection of asymptomatic carriage, quantitative thresholds to differentiate symptomatic, and asymptomatic infections have been estimated for some enteropathogens [63]; (3) inability to distinguish between viable and nonviable microorganisms [72,73]; (4) they do not offer antibacterial susceptibility or epidemiological data; these aspects can be solved including specific cultures when a bacterial enteropathogen is detected in the panel, and once the bacterium is isolated susceptibility testing and speciation and serotyping, for instance for *Salmonella* and *Shigella*,

Table 2. Main advantages and disadvantages of using multiplex molecular assays.

Advantages	Disadvantages
Increased diagnostic yield	Some microorganisms can be shed in feces for several weeks, which may not be clinically relevant
Workflow is improved	Detection of asymptomatic carriage
Reduced hands on time	Not distinguish between viable and nonviable microorganisms
Impact on infection control	They do not normally offer antibacterial susceptibility or epidemiological data
Shorter hospital stay and reduction of antibiotic therapy	Cost is the main current concern

can then be performed; (5) a wide range of different commercial panels is available, frequently requiring specific instruments for the performance of the test or the detection of reaction products, making it necessary for laboratories to invest in several different molecular platforms; and (6) the high cost making the analysis of all the samples difficult to implement, taking into consideration that between 40% and 50% of the samples are negative, mainly those of community-acquired gastroenteritis or traveler's diarrhea. Therefore, the application of these tools should be performed in selected patients likely based on the severity of the gastroenteritis or the clinical condition of the patient.

Five-year view

Gastroenteritis may not always be severe and may often resolve rapidly. Nonetheless, gastrointestinal infections may be serious in specific health-care settings or patient populations, such as newborns/infants, the elderly, or immunocompromised patients. In addition, several enteropathogens can cause rapidly spreading outbreaks and epidemics. Therefore, rapid diagnosis is particularly important in these public health contexts. In the near future, the increase in the aging population and the advances in treatments for patients with hematological, oncologic, and rheumatologic diseases will lead to an increase in the number of immunocompromised patients presenting to primary care and general hospitals with opportunistic infections. Therefore, a rapid test to detect the cause of the diarrhea is crucial for these specific patients. In addition, another important application of these rapid molecular tests is to distinguish diarrhea as a symptom of host versus graft disease from that caused by a microorganism.

Based on this scenario, two situations can be visualized; the first being a patient with a severe diarrhea attending the emergency department or admitted to the hospital with a need of a very rapid test. In this case, an integrated platform is ideal since it includes most of the pathogens, requires less hands-on time, and is more user friendly and highly trained personnel are not needed. The second situation is that of a patient with mild or moderate acute or chronic diarrhea. In this case, a less costly approach can be performed.

Looking toward a more distant future, whole genome-next generation sequencing (WG-NGS) will provide further information mainly when applied to samples that remain negative by either routine or multiplex-PCR-based diagnostic methods and the patient is still symptomatic. This approach will allow the identification of non-predefined targeted microorganisms. The implementation of WG-NGS technology as the first approach will ultimately depend on the ratio between costs and clinical benefits.

Key issues

- Multiple enteropathogens (bacteria, viruses, and parasites) can cause gastroenteritis.
- The etiological agent of between one-third or two-third of gastroenteritis remains unknown.
- Conventional methods used to detect enteropathogens are culture, microscopy, antigen test.
- Different PCR-based panels have been developed to detect the microorganisms that often causing gastroenteritis.
- The PCR-based assays show a high specificity and sensitivity.
- Although many studies on cost-effectiveness of the incorporation of these assays in the routine analysis are not available, they would be useful for rapid detection of etiological agents and improvement in patient care.
- The assays which are quantitative have been found to be useful in differentiating clinically significant infections.
- Specific culture of bacterial enteropathogens is required to study their epidemiology and susceptibility to antibiotics.
- The selection of samples in which this molecular methodology can be used should be adapted based on clinical, epidemiological, and microbiological criteria.

Declaration of interest

The authors thank the Ministerio de Economía y Competitividad, Instituto de Salud Carlos III – co-financed by the European Development Regional Fund 'A way to achieve Europe' ERDF, Spanish Network for the Research in Infectious Diseases (REIPI RD12/0015) and FIS 14/0755. This study was also supported by grant 2014SGR0653 from the Departament d'Universitats, Recerca i Societat de la Informació of the Generalitat de Catalunya.

The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.

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