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Multiplex PCR Tests for Detection of Pathogens Associated with Gastroenteritis

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Synopsis

A wide range of enteric pathogens can cause infectious gastroenteritis. Conventional diagnostic algorithms including culture, biochemical identification, immunoassay and microscopic examination are time consuming and often lack sensitivity and specificity. Advances in molecular technology have as allowed its use as clinical diagnostic tools. Multiplex PCR based testing has made its way to gastroenterology diagnostic arena in recent years. In this article we present a review of recent laboratory developed multiplex PCR tests and current commercial multiplex gastrointestinal pathogen tests. We will focus on two FDA cleared commercial syndromic multiplex tests: Luminex xTAG GPP and Biofire FimArray GI test. These multiplex tests can detect and identify multiple enteric pathogens in one test and provide results within hours. Multiplex PCR tests have shown superior sensitivity to conventional methods for detection of most pathogens. The high negative predictive value of these multiplex tests has led to the suggestion that they be used as screening tools especially in outbreaks. Although the clinical utility and benefit of multiplex PCR test are to be further investigated, implementing these multiplex PCR tests in gastroenterology diagnostic algorithm has the potential to improve diagnosis of infectious gastroenteritis.

Keywords

gastroenteritis; multiplex PCR; laboratory developed test

Introduction

Infectious gastroenteritis still presents itself as a worldwide health problem with an estimated 2 billion cases of diarrhea that occur yearly and causes over 2 million deaths every

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year[1–4]. For children under 5 years of age, infectious gastroenteritis or diarrhea is the leading cause of death worldwide with an estimated 1.9 million death of children every year[1, 5, 6]. In 2010, there were estimated 1.731 billion episodes of diarrhea (36 million of which progressed to severe episodes) [7]. In the United States acute gastroenteritis (AGE) is a major cause of illness with an estimated 179 million episodes annually[8]. A report from Lucado et al. infectious enteritis and foodborne illness in the United States during 2010 found that nearly 1.3 million inpatient hospital stays had diagnoses of enteritis or gastrointestinal symptoms[9].

A wide range of enteric pathogens cause infectious gastroenteritis. Common causative agents include bacteria such as *Campylobacter, Salmonella, Shigella, Clostridium difficile* and pathogenic *E. coli* [10, 11]; viruses such as norovirus, rotavirus and adenovirus serotypes 40 and 41[12–14]; and parasites such as *Giardia*, *Entamoeba histolytica* and *Cryptosporidium*[15, 16]. Early identification of the causative pathogen is still a challenge in the clinical laboratory. Stool culture is the primary diagnostic tool for suspected bacterial infection. Usually selective agars and subsequent morphologic, biochemical and serologic testing are required in order to identify and confirm the suspected culture isolate. Stool-culture based diagnosis is time-consuming, labor-intensive and costly on per positive culture basis[17–21]. In addition to this low culture positive yield, there is a significant variability between physicians in the likelihood that a stool culture is requested for a given patient[22]. Inconsistent testing leads in parts to inconsistent treatment, such as the use of antimicrobial therapy. Rapid and accurate identification of the infectious agent will allow clinicians to choose the appropriate antimicrobials if needed or to avoid them if they are not indicated.

Most viruses that cause infectious gastroenteritis cannot be cultured. Methods such as electron microscopy (EM) examination and immunassays have been used for viruses such as norovirus, rotavirus and adenovirus. These methods require significant expertise, are labor-intensive, and can be subjective in interpretation. Microscopic examination is still routinely used for parasite identification. An ova and parasite examination requires a well-trained technologist to examine the prepared specimen. Because the examination is mainly focused on morphology, the interpretation is subjective. Furthermore, in some cases one cannot distinguish different species; for example *Entamoeba histolytica* (pathogenic) and *Entamoeba dispar* (non-pathogenic)[23]. Clearly rapid and accurate diagnostic tools are needed for infectious gastroenteritis.

Emerging molecular diagnostic tools

Emerging diagnostic assays have started to make their way into the microbiology, virology and parasitology laboratories in gastroenterology. These tests address limitations of conventional diagnostics by taking advantage of technological advances.

Molecular tests for various enteric bacteria pathogens have been developed and are sold by multiple companies. The evolution of molecular diagnostic assays for detection of toxigenic *Clostridium difficile* illustrates the availability of different molecular technologies and the adoption of molecular testing for a GI pathogen. Since January 2009, the Food and Drug Administration (FDA) has approved several in vitro diagnostic (IVD) molecular assays for

detection of toxigenic *C.difficile*. Most of these assays offer rapid turn around time (<2hr) with minimal hands-on time, and high sensitivity and specificity compared to toxigenic culture. Key features and applications of the *C.difficile* assays are provided in the review by Svensson AM et al. and Dunbar SA et al[24, 25].

New diagnostic tools such as enzyme immunoassay (EIA) and molecular tests have shown a much higher positive rate of *Campylobacter* infection than found by culture[26]. Most laboratories use selective culture techniques for isolation of *Campylobacter jejuni* and *C. coli*, which led to the thought that they are the primary species associated with gastroenteritis. The study reported by Platts-Mills used EIA and PCR tests for Campylobacter infection in children with diarrhea in the developing world. The authors have shown a higher rate of *Campylobacter* infection using these methods[27].

Molecular diagnostic tools for detection of gastrointestinal viruses, such as norovirus, are emerging rapidly. In April 2014 Cepheid Xpert® Norovirus marketed as a CE IVD product under the European Directive on *In Vitro* Diagnostic Medical Devices. Cepheid Xpert® Norovirus is a qualitative test for rapid identification and differentiation of Noroviruses genogroup I (GI) and genogroup II (GII). The assay is claimed to provide high sensitivity (100% GI & GII) and high specificity (99.5% GI & 98.9% GII) with a total turn around time of <1hr[28]. A PCR-based diagnostic test for norovirus was used for a study on global prevalence of norovirus in gastroenteritis[29]. The study showed that Norovirus is a key gastroenteritis pathogen associated with almost a fifth of all cases of acute gastroenteritis. In addition, noroviruses are a major cause of closure of hospital wards, and are associated with increased hospitalization and mortality among the elderly. Transplant patients have significant risk of acquiring persistent norovirus gastroenteritis. The emerging molecular tests for noroviruses lead to the increased recognition of its significance in gastroenteritis and calls for antiviral treatment and prophylaxis of norovirus infections, and possibly vaccination[30].

Detection and identification of GI pathogens in a multiplex format

The most exciting emerging technologies are using multiplex molecular assays or pathogen class specific multiplex molecular assays for comprehensive syndromic gastrointestinal pathogen detection. In the past few years with the advances in molecular technologies along with the improved technologies for sample preparation and nucleic acid extraction/ purification, multiplex RT-PCR based detection has gradually made its way into diagnostic laboratories.

The advantages of testing one sample for multiple common pathogens using a single test are 1) to reduce the turnaround time for accurate results; 2) to identify co-infections; 3) to utilize the high negative predictive value of a multiplex assay to cease unneeded infection control precautions; 4) to benefit from the high sensitivity and specificity that most molecular testing offers; and 5) to help health care providers provide better care to patients.

Multiplex molecular detection of respiratory virus nucleic acid has revolutionized the routine laboratory diagnosis of viral infections since the first multiplex testing for respiratory

viruses, xTAG® Respiratory Virus Panel (RVP) (Luminex Corporation, USA) received FDA clearance in January 2008. Over the past 6 years, there have been more than 9 commercial molecular diagnostic assays for the detection of respiratory viruses. The utility of such assays, advantage and disadvantages has been studied and reviewed extensively[31–36]. With the promises seen in respiratory multiplex testing, efforts have been made to demonstrate the utility of multiplex molecular testing utility in gastrointestinal infections.

In the recent study by Liu J et al the performance and benefit of three multiplex molecular platforms for the detection of 15 enteropathogens were evaluated against conventional comparator methods (bacterial culture, ELISA and PCR) using over 1500 clinical samples across five laboratories worldwide[37]. Liu's study showed that laboratory-developed PCR-Luminex assay, multiplex real-time PCR and TaqMan array card assays cost US $25–60 per sample. When using seven ELISA kits, from different companies, three types of culture media and various biochemical reagents in order to test different enteropathogens, the total reagent cost per sample was about $200. In addition to the cost savings from multiplex molecular testing, the turnaround time for multiplex syndromic panel testing takes hours or days for testing 100 samples instead of weeks for conventional comparator methods[37, 38].

There are two main categories of multiplex molecular testing. One category is known as syndromic panels. A multiplex syndromic panel usually includes most common pathogens responsible for a particular array symptom. For example a multiplex panel for diarrhea may test for the most common causative agents from various pathogen classes such as bacteria, viruses and parasites. Luminex xTAG Gastrointestinal Pathogen Panel (GPP) and Biofire's FilmArray Gastrointestinal (GI) Panel are such syndromic panels. The other category of multiplex molecular assays includes a class of pathogens in a multiplex panel. Becton Dickinson's BD MAX System has three products for enteric pathogens, each panel targets one class of pathogen, BD Max System Enteric Bacterial Panel, Parasite Panel and Viral Panel.

Multiplex testing for gastroenteritis is still in its infancy comparing to multiplex testing for respiratory tract infection. Using multiplex detection and identification of gastrointestinal pathogens has made noted progress in the past couple of years. It is anticipated that multiplex testing will increase diagnostic positivity, identify co-infections, faster turnaround time and may reduce the use of antibiotics. There are multiplex RT-PCR based laboratory developed test (LDT) and commercial assays (mostly not FDA approved yet) available for gastrointestinal pathogen testing[25, 39]. As more multiplex testing becomes available, the value of using such a test will be assessed and recognized. Evaluations of xTAG GPP by Wessels et al. demonstrated the added value of this multiplex test in terms of the increased positivity rate, one test with multiple answers and proving results within one-day shift[40]. The next sections introduce and discuss current GI research multiplex assays and commercial multiplex assays.

Current multiplex GI research assays

Research developed assays have been in the forefront in the application of advanced molecular detection technology for research and or diagnostic use in laboratories. This

section provides examples of LDTs that use multiplex molecular testing for gastroenteric pathogens. Table 1 lists the published LDTs that are discussed here, institutions and pathogens that these tests detect.

Milwaukee Health Department Laboratory developed and validated a 19-plex laboratory-developed gastrointestinal pathogen panel (GPP) using Luminex xTAG analyte-specific reagents (ASRs)[41]. This laboratory developed test can simultaneously screen for diarrhea-causing pathogens, including 9 bacteria (*Campylobacter jejuni, Salmonella* spp., *Shigella* spp., enterotoxigenic *Escherichia coli* [ETEC], Shiga toxin-producing *E. coli* [STEC], *E. coli* O157:H7, *Vibrio cholerae, Yersinia enterocolitica*, and toxigenic *Clostridium difficile*), 3 parasites (*Giardia lamblia, Cryptosporidium* spp., and *Entamoeba histolytica*), and 4 viruses (Norovirus GI and GII, Adenovirus 40/41, and Rotavirus A) directly from fecal specimens. The evaluation study of this multiplex LDT included 48 reference isolates and 254 clinical specimens. The overall comparative performance of the multiplex test with conventional methods in clinical samples was 94.5% (range, 90% to 97%), with 99% specificity. The study result indicated that this multiplex assay enables sensitive and specific screening and identification of the major acute diarrheal pathogens.

Another Luminex platform-based multiplex PCR assay was developed by Jiu J et al from Division of Infectious Diseases and International Health, University of Virginia[37]. The multiplex RT-PCR assay detects 15 common enteropathogens including 5 viruses (Adenovirus, Astrovirus, Norovirus GII, Rotavirus, and Sapovirus), 7 bacteria (*Campylobacter jejuni /C. coli, Salmonella* spp, *Vibrio cholerae*, enteroaggregative *E. coli* [EAEC], enterotoxigenic *E. coli* [ETEC], enteropathogenic *E. coli* [EPEC], *and* enterotinvasive *E coli* (EIEC), and 3 parasites (*Cryptosporidium* spp, *Giardia* spp, *and Entamoeba histolytica*). The assay was used in a multicenter study that showed molecular tests can be deployed successfully in different parts of the world and detected enteropathogens with high sensitivity and specificity, and identified mixed infections[37].

Nanolitre real-time PCR panel is another novel technology that allows the user to carry out multi-target panel in using very low volumes. This technology is a high-throughput quantitative real-time reverse transcriptase (RT)-PCR platform that can perform over 3,000 separate PCR reactions in parallel in 33 nanolitre volumes in through-holes (similar to wells on a microtitre plate). A multiple-target nanolitre realtime PCR panel was developed for 16 major diarrhoeal pathogens by Goldfarb DM et al[42]. This panel detects:

8 bacteria:

1) Enterohemorrhagic *Escherichia coli* (EHEC), via detection of *stx* 1, *stx* 2, *E. coli* O157
2) *Salmonella* spp.
3) *Shigella* spp.
4) *Campylobacter* spp.
5) Yersinia entercolitica
6) *Clostridium difficile, Clostridium difficile tcd* B

7) Listeria monocytogenes

8) Vibrio parahaemolyticus

6 viruses:

1) Norovirus group 1

2) Norovirus group 2

3) Rotaviruses

4) Astroviruses

5) Adenoviruses 40/4

6) Sapoviruses

and 2 parasites:

1) Giardia lamblia

2) *Cryptosporidium* spp.

This nanolitre real-time PCR panel was used to test stool samples collected from Canada's Arctic region, Qikiqtani (Baffin Island) Region of Nunavut. This PCR based assay detected *Cryptosporidium* spp that was missed by microscopy or enzyme immunoassay.

A number of multiplex molecular LDTs have been developed for the detection of gastrointestinal viruses over the years. Khamrin et al from Department of Microbiology, Faculty of Medicine, Chiang Mai University, Thailand, has developed a single-tube multiplex PCR for the detection of 10 viruses, including rotaviruses group A and C, adenovirus, norovirus GI, norovirus GII, sapovirus, astrovirus, Aichi virus, parechovirus, and enterovirus. Upon evaluation of this novel 10-multiplex viral panel against a total of 235 stool samples collected from infants and children with acute gastroenteritis, 111 of the 235 (47.2%) stool samples were positive for a pathogen. The study suggested that this multiplex PCR is useful as a rapid and cost effective diagnostic tool for the detection of major pathogenic viruses causing diarrhea[43].

Similarly, Pang et al from Provincial Laboratory for Public Health, Edmonton, Alberta, Canada developed a multiplex real-time PCR panel (EVPrtPCR) that simultaneously detects 5 enteric viruses (EV) including rotavirus, norovirus, sapovirus, astrovirus, and enteric adenovirus in stool samples[44]. In the study reported by Pang et al, a total of 2,486 sporadic gastroenteritis samples submitted for EV testing using electron microscopy (EM) between July 2008 and July 2009 were tested with this EVPrtPCR panel. The real-time assay detected 30% more viruses than that identified by EM.

In 2011, Regional Virus Laboratory of Royal Victoria Hospital, Belfast, Northern Ireland has developed and validated a multiplex TaqMan assay for the detection of viral gastroenteritis[45]. The assay probes for 4 different viruses, rotavirus, norovirus (genogroups I and II), astrovirus, and adenoviruses (serotypes 40 and 41). In a validation study using 137 specimens, the assay showed sensitivity for adenovirus of 97.3%, rotavirus of 100%, and norovirus of 95.1% and specificity for adenovirus of 99%, rotavirus of 100% and

adnorovirus 97.9%. Astrovirus gave 100% sensivitity and specificity with the samples tested. The assay has been successfully used in routine diagnostic services.

Multiplex LDT for the detection of various bacterial enteric pathogens have been developed by various laboratories. A multiplex PCR/ligation detection reaction (LDR) assay was developed by Rundell MS et al for the detection of bacterial pathogens from stool specimens[46]. The panel targets seven bacterial pathogens, *Campylobacter* spp., *Vibrio* spp., *Shigella* spp., *Salmonella* spp., *Listeria monocytogenes, Yersinia enterocolitica, and* diarrheagenic *Escherichia coli*. The sensitivity and specificity of the assay were assessed using primarily contrived samples (cultured-negative stool specimens spiked with known isolates) and a small number of clinical specimens from Haiti. The overall sensitivity ranged from 91% to 100% and the overall specificity ranged from 98% to 100% depending on the species. The study showed that it is feasible to use a PCR/LDR multiplex assay for the detection of a panel of enteric bacterial pathogens.

A five-gene panel was recently developed by Al-Talib H et al for the identification of the common hemorrhagic bacteria in stool samples[47]. Specific primer sets were designed for ompC of *Salmonella* genus, virA gene for *Shigella* genus, eaeA gene for enterohemorrhagic *Escherichia coli* (EHEC), 16S rRNA for *Campylobacter* genus, and hemA for an internal control. In the study carried out by Al-Talib et al, this one-tube multiplex PCR assay had a limit of detection of 1×10^3 CFU at the bacterial cell level and 100 pg at the genomic DNA level. Evaluation with 223 bacterium-spiked stool specimens showed 100% sensitivity and specificity. The assay has a 4-hr turnaround time for the identification of hemorrhagic bacteria.

Multiplex molecular testing not only applies to detect multiple different pathogens, it is also used to detect and distinguish different subtypes for a given organism. A multiplex PCR assay was developed by Chankhamhaengdecha S et al. to detect various *C. difficile* ribotypes, other *Clostridium* spp., and non-*Clostridium* strains by targeting different toxin genes: tcdA, tcdB, cdtA, and cdtB[48]. The study showed 100% specificity with the ability to detect as low as ~22 genomic copy number per PCR reaction.

Research laboratory developed multiplex assays have been used in other applications. For example, Heidary M et al developed a multiplex PCR assay for the detection of antibiotic resistance genes for diarrheagenic *Escherichia coli* [49]. Wang XG et al has explored multiplex PCR approach for 6 non-O157 STEC virulence genes detection and showed that the multiplex PCR tests had comparable results to serological testing[50]. In summary, research or laboratory developed multiplex molecular tests have been used for gastroenteritis research and clinical diagnostic in certain institutions. It should be noted that the laboratory developed multiplex tests mentioned and listed in this article (Table 1) are examples to illustrate the advancement of molecular testing in gastroenterology and there may be many other similar molecular tests developed by various laboratories over the years.

Current Enteric Multiplex Commercial Assays

The review for gastrointestinal (GI) multiplex commercial assays includes two parts. Part 1 is an overview of 13 currently available GI multiplex commercial assays, and Part 2 is a detailed discussion on the two FDA cleared syndromic multiplex assays: Luminex xTAG GPP and BioFire FilmArray GI Panel. Table 2 provides a detailed breakdown of the 13 commercial multiplex assays: manufacture, test name, US-IVD (FDA cleared) or CE-IVD, number of GI pathogens detected, time to result, throughput, sample processing / extraction, specimen type, assay technology, specialized equipment requirement and assay complexity. Table 3 lists pathogens that are detected by each of these multiplex assays.

Overview of multiplex commercial assays

Over the past few years the gastroenterology diagnostic market has witnessed a surge of commercial multiplex diagnostic assays for the detection of various gastroenteric pathogens. These multiplex commercial assays differ in many aspects, including the number of different pathogens that the assay detects, throughput of the assay, overall time to results, regulatory status, and the complexity of the assay. Of the 13 multiplex commercial assays, only 3 assays are considered as comprehensive syndromic (virus, bacteria and parasite) multiplex RT-PCR based assays. The other 10 commercial assays are multiplex assays that allow detection of members of a specific class of pathogens.

Syndromic GI multiplex assays—Luminex's xTAG GPP was the first syndromic GI assay that received FDA clearance[51] and was CE-marked in May 2011. xTAG GPP is based on multiplex RT-PCR for target amplification and detection using Luminex microsphere xMAP and xTAG technologies. The assay is approved for the detection of 14 pathogens (8 bacteria, 3 viruses and 3 parasites)[52].

The FilmArray GI Panel produced by Biofire is a self-contained system using PCR with melt analysis of the PCR product for analyte detection. The FilmArray GI Panel received FDA clearance for the detection of 20 pathogens (11 bacteria, 5 viruses and 4 parasites)[53]. Both xTAG GPP and FilmArrays GI panel are discussed in Part 2.

The Gastrofinder Smart 17 Fast, produced by PathoFinder, is a non-FDA cleared but CE-marked realtime PCR based multiplex GI assay that allows the detection of 17 pathogens (9 bacteria, 4 viruses and 4 parasites). The assay does not require specialized equipment. Sample preparation is performed using standard commercial available nucleic acid purification techniques and RT-PCR/detection is achieved using a standard real-time thermal cycler. The high level of multiplexing on a standard real-time thermal cycler is achieved using Pathofinder's SmartFinder technology in which hybridization probes are ligated together to create amplimers of varying length that can be differentiated by melt curve analysis. Limited performance data is available for the GastroFinder assay. One study of 120 retrospective clinical samples found that the sensitivity ranged from 60% - 100%; specificity information was not provided[54].

Commercial multiplex assays for specific pathogen classes—Of the ten commercial assays, three assays have received FDA clearance: Nanosphere Verigene Enteric

Pathogen test (June 2014 clearance), the BD MAX Enteric Bacterial assays (May 2014 clearance) and the Hologic ProGastro SSCS (January 2013 clearance). The Nanosphere Verigene Enteric Pathogen test is a two-step automated platform covering 6 bacterial and 2 viral targets. A Cary-Blair stool sample is loaded into a cartridge on a sample processing unit which extracts and amplifies the target nucleic acid. The Amplified nucleic acid is automatically transferred to a microarray which uses gold nanoparticle probes and a silver staining process to generate signal. The microarray is then analyzed using the Verigene reader system. The sensitivity for the Verigene system from prospectively collected samples evaluation ranged from 67% – 100% while the specificity ranges from 99% – 100%[55].

The BD MAX Enteric bacterial Panel received FDA clearance for detecting four bacterial targets from raw stool or stool in Cary-Blair transport medium. The assay runs on the BD MAX microfluidic automation platform which incorporates sample preparation and microfluidic Real-time PCR detection into one system. The system can simultaneously process up to 24 samples in four hours. Based on FDA 510(k) summary, the sensitivity of the BD MAX Enteric bacterial Panel ranged from 85% - 100% while the specificity ranged from 98% - 99% when tested with 3457 patients samples suspected of acute bacterial gastroenteritis, enteritis or colitis[56]. BD has also developed an Enteric Parasite panel that detects 3 parasitic targets from raw stool or formalin fixed stool. At the time of this review the BD Enteric Parasite panel is CE-IVD (May 2014) but not FDA approved. A unique aspect of the BD MAX system is that it is an open platform which allows other assay manufactures or laboratories to develop assays to run on the platform.

The third FDA cleared panel for the detection of bacterial pathogens is Hologic ProGastro SSCS panel. The assay detects 4 bacterial pathogens from stool in Cary-Blair transport medium in a two-tube TaqMan based real-time PCR assay. The ProGastro SSCS assay runs on a standard real-time thermal cycler (e.g. Cepheid SmartCycler). Nucleic acid from Cary-Blair stool samples can be extracted and purified using bioMerieux NucliSENS easyMAG extractor. The sensitivity of the assay ranged from 95% to100% with a specificity of 99% as reported in the 510k summary[57]. In a separate study the ProGastro SSCS assay was compared to culture for the identification of *Campylobacter* spp. (*Campylobacter jejuni* and *Campylobacter coli*), *Salmonella* spp., and *Shigella* spp. and to broth enrichment followed by an FDA-cleared enzyme immunoassay (EIA) for the identification of shiga toxin-producing *Escherichia coli* (STEC) isolates in stool specimens. When results based on the ProGastro SSCS assay and bidirectional sequencing for discrepancy analysis were compared to conventional testing, the sensitivity of the ProGastro SSCS assay was 100% for all pathogens, and the specificities ranged from 99.4% to 100%[58].

The rest of the seven multiplex assays have not received FDA clearance. These assays detect for two to four pathogen targets (low/moderate-plex) per reaction well. Although these assays do not provide the same level of multiplexing as some of the other GI diagnostics, they typically provide the user with more choice in terms of which pathogens they wish to test for. Usually the low/medium-plex assay manufacturers create several assays, some of which can be run concurrently. The majority of the low/medium-plex assays are based on multiplex Real-time PCR detection; yet some of these assays differ in sample preparation / extraction step, assay reagents and signal detection mechanisms.

Serosep's EntericBio Gastro Panel is unique in that it does not require traditional nucleic acid purification; instead the stool samples are incubated with a sample preparation buffer followed by heating at 97°C f or 30 min to release bacterial DNA. The cooled sample is then added directly to lyophilized PCR reagents. The sample processing and PCR setup of the Serosep assay can be performed with an epMotion liquid handler which results in very little hands on time[59]. The EntericBio real-time Gastro Panel I for simultaneous detection of *C. jejuni, C, coli*, and C. *lari*, Shiga toxin-producing *Escherichia coli (STEC), Salmonella* spp., *and Shigella* spp.was evaluated by Koziel M et al with a total of 528 prospectively collected samples from patients with acute gastroenteritis[60]. The assay reported 84 positive results, including *Campylobacter spp*. (n=44); 35 Stx1/Stx2 (n=35); *Shigella* spp. (n=3); and *Salmonella* spp. (n=6). Comparing to a previous version of this assay and culture results from retrospective samples, the sensitivity and specificity of the assay was reported as 100% and 97.8% respectively.

The sample preparation is also unique for Genetic Signature's EasyScreen Enteric assays (Sydney, Australia) which uses the company's 3base technology to convert all cytosine bases (C) in the starting nucleic acid sample to thymine (T). The resulting reduction in sequence variation allows for a higher number of multiplex targets to be run under similar conditions[61]. This universal sample processing technology was evaluated by Siah et al with 487 characterized stool samples representing bacterial, viral, protozoan and *Clostridium difficile* positive samples. The processed samples were subsequently tested using four multiplexed real time PCR panels. The study suggested that these multiplex real time PCR panels with universal sample preparation generated comparable results as conventional methods, but with the added advantage of streamlined and rapid diagnosis of gastrointestinal pathogens[62]. One study was reported on EasyScreen™ Enteric Parasite Detection Kit for the identification of 5 common enteric parasites: *Blastocystis* spp., *Cryptosporidium* spp., *Dientamoeba fragilis*, *Entamoeba* complex, and *Giardia intestinalis* in human clinical samples. When compared to real-time PCR and microscopy, the EasyScreen™ Enteric Parasite Detection Kit showed 92–100% sensitivity and 100% specificity upon testing a total of 358 stool samples[63].

The workflows for R-BioPharma AG (Darmstadt, Germany), Fast-Track Diagnostics (Sliema , Malta) and Diagenode assays all follow the tradition real-time PCR workflow of nucleic acid purification, PCR setup and amplification/detection[64–66]. Similarly, these companies offer specific pathogen panels of low/moderate plex capacity per panel. A recent study by Biswas JS et al evaluated and compared the diagnostic accuracy; turnaround time and ease of use of three multiplex molecular panels: the RIDA®GENE Bacterial Stool and EHEC/EPEC Panels, the FTD® Bacterial Gastroenteritis and the BD MAX™ Enteric Bacterial Panel. The study tested the three panels with 116 retrospective samples and 318 prospective stool samples. Conventional culture-based techniques and consensus among molecular assays were used as the gold standards. A positive test was based on either culture positive or agreement in two of the three molecular panels. The three multiplex molecular panels were more sensitive than culture for most of the targets, detecting an additional 13 cases that were culture negative. All three molecular panels gave much faster turnaround time than culture: <3 h vs 66.5 h for culture. The BD MAX™ panel was noted as the fastest, easiest to use and most flexible[67].

Genomica has developed a two-tube CLART EnteroBac panel that detects 7 analytes. The assay follows the traditional molecular workflow for sample extraction and amplification; however, detection is carried out on a low-density microarray which is analyzed by the company's CAR reader. A unique feature of the CLART EnteroBac panel is that it differentiates some of the *campylobacter* and *Yersinia* species[68].

AusDiagnostics Faecal Bacteria and Gastrointestinal Parasite panels step away from the traditional real-timer PCR workflow with their Multiplex Tandem PCR technology. In this PCR system, extracted nucleic acid is pre-amplified in a single well multiplex PCR reaction, then several aliquots of the pre-amplification material are transferred into a singleplex intercalating dye real-time PCR reaction where amplification and melt-curve analysis is used to detect the present targets[69]. This multiplex tandem PCR (MT-PCR) based assay was developed for the detection of 4 protozoan parasites, (*Cryptosporidium* spp., *Dientamoeba fragilis*, *Entamoeba histolytica* and *Giardia intestinalis*). This 4-plex assay was evaluated with 472 fecal samples. When using singleplex real-time PCR as comparator, this 4-plex MT-PCR assay had 100% sensitivity and specificity. The traditional microscopy examination only gave a sensitivity of 38%~56%, highlighting the superior sensitivity of molecular testing[70].

Seegene's Seeplex Diarrhea ACE assays use the company's dual priming oligonucleotide technology to provide increased specificity during PCR amplification. The dual priming oligonucleotide technology is based on placing a poly-deoxyinosine linker near the 3' end of the primer which slightly destabilizes it, lowering the chance of non-specific priming. The Seeplex Diarrhea assay also differs from the other assays in that it uses auto capillary electrophoresis for detection of the amplified products[71]. There are two patient cohort based evaluation studies on Seeplex Diarrhea ACE assays. One study was by Coupland LJ et al to evaluate Seeplex Diarrhea ACE multiplex detection for four viruses and/or ten bacteria with 223 patients' samples[72]. Comparing to conventional methods and Norovirus-specific RT-PCR, Seeplex Diarrhea ACE panels showed 100% positive concordance for Adenovirus, Norovirus, *Campylobacter* spp., *Escherichia coli* O157, *Shigella* spp. *or Vibrio* spp. The ACE panels missed 12.5% of Rotavirus, 50% of *Clostridium difficile* toxin B, and 15.8% of *Salmonella* spp. of the positive samples. The second study was conducted with 245 pediatric patients using Seeplex Diarrhea ACE assays (ACE-Bacteria 1, Bacteria 2 and Viral assays) collectively detecting 15 enteric pathogens, including *Salmonella* spp., *Shigella* spp., *Vibrio* spp., toxin B producer *Clostridium difficile, Campylobacter* spp., *Clostridium perfringens, Yersinia enterocolitica, Aeromonas* spp., *Escherichia coli* O157:H7, verocytotoxin-producing *Escherichia coli*, Adenovirus, group A rotavirus, Norovirus GI and GII, and Astrovirus[73]. This study showed better sensitivity for multiplex PCR than routine methods, except for *Salmonella* spp. and toxigenic *C. difficile*.

FDA cleared syndromic multiplex assays: Luminex xTAG GPP and BioFire FilmArray GI Panel

Luminex xTAG GPP—Luminex's xTAG GPP assay was the first large multiplex syndromic panel that received FDA 510(k) clearance (January 2013) for the detection of gastroenteritis causing pathogens including bacteria, viruses and parasite. With the recent

September 2014 subsequent clearance, xTAG GPP can be used to detect 14 most common pathogens (8 bacterial, 3 viral and 3 parasitic pathogens from a single sample) in both raw stool specimen and stool in Cary-Blair media. The assay is based on Luminex's xTAG and xMAP technologies. The workflow of the assay starts with a sample pretreatment step with bead beating in order to breakthrough parasitic pathogen followed by nucleic acid extraction/purification, and a single multiplex RT-PCR followed by bead hybridization and detection.

The bead beating step in the pretreatment is required for any lysis-resistant parasites such as *Cryptosporidium* oocysts. This step is performed by adding ~100 mg of stool, 100 μL of liquid stool or 400 μL of stool in Cary-Blair medium to a Bertin SK38 bead tube to which NucliSENS easyMAG lysis buffer and the internal control (xTAG MS2) are also added. The bead tube is then vortexed for 5 minutes, incubated at room temperature for 10 minutes and followed by a brief centrifugation. The volumes of input stool vary between plain stool and stool in Cary-Blair due to the dilution factor that occurs when a stool sample is placed in Cary-Blair medium. To minimize PCR inhibition occurrence, appropriate amount of stool input is important (ie not to add too much stool specimen). Wessels *et al*. noted that reducing the amount of stool input into the assay reduced the PCR inhibition rate from 7.6% to 2.3%[40]. The overall rate of PCR inhibition (as determined by an internal control failure) ranges from as high as 14% - 16%[52] to ~2.3% and 7.7%[40, 74]. It is important to note that when the internal control is inhibited a positive analyte call can still be made if an analyte produces a positive signal; however, the analytes cannot be identified as negative if no internal control signal is obtained. It is recommended that the purified nucleic acid from inhibited samples be diluted 1:10 and rerun the assay. This dilution procedure has been shown to recover > 80% of inhibited samples[40, 75].

The xTAG GPP US-IVD product insert recommends that nucleic acid extraction be performed using a bioMerieux NucliSENS easyMAG running the specific A 1.0.2 protocol. Under this protocol 200 μL of pretreated material is used with a elution volume of 70 μL of purified nucleic acid (extraction run takes approximately 55 minutes). However, the xTAG GPP CE-IVD product insert also states that the QIAamp MinElute Virus Spin Kit by Qiagen can be used. In addition to the two recommended nucleic acid extraction/purification platforms, end users have applied other extraction methods and platforms with xTAG GPP. Other extraction platforms include Roche's MagNA Pure[75]. Qiagens EZ1 virus mini kit[76], Qiagen's QIAsymphony[40], and Abbott m2000sp instrument[77].

The multiplex RT-PCR setup for the Luminex xTAG GPP assay follows a standard molecular workflow. It is recommended that all RT-PCR reactions setup be performed on cold blocks or PCR coolers, to prevent non-specific activity of the reverse transcriptase. 10 uL of purified nucleic acid is added to the reaction and it placed on a standard end-point thermal cycler. The thermal cycling takes approximately 2 hours and 10 minutes. In the product insert Luminex highlights the importance of maintaining a clean pre-PCR area. Because the xTAG GPP assay is an open system that requires handling of amplified material in the hybridization step, the risk of contamination should be noted.

The xTAG GPP hybridization and detection reaction is a liquid phase reaction where amplified RT-PCR product is combined with the xTAG GPP bead mix and the fluorescent reporter streptavidin r-phycoerythrin (SAPE). During the hybridization reaction, tags on the amplified RT-PCR product hybridize with their complement tag on the Luminex microspheres and the SAPE reporter binds to the biotin on the amplified product. The signal detection and data acquisition is obtained by a Luminex 100, Luminex 200 or MAGPIX instrument where the sample is read. Data analysis is performed by the xTAG GPP TDAS software. The TDAS software provides result and report with one of three outcomes for each sample POS (positive), NEG (negative) or No Call. The No Call result is given when one of the assay parameter is not met or there is an internal control failure. An important aspect of the TDAS software is that it allows flexibility for end users to only select the analytes that they wish to detect, which in turn masks those results that the end users do not wish to see.

The FDA 510(k) clinical study of xTAG GPP reports an overall sensitivity of 80.0% - 100.0% for all analytes with the exception of Enterotoxigenic *E. coli* (ETEC). The sensitivity for ETEC was 25.0% (2/8); the 6 ETEC samples that were reported as false negative by xTAG GPP were tested using four other well characterized NAAT's, only 1 of the 4 NAAT's called the 6 samples positive. The specificity of the xTAG GPP assay ranged from 89.8% - 99.9%, with a negative predictive value of >99%. Despite this relatively high level of specificity the US-FDA placed a "presumptive positive" warning on the xTAG GPP assay requiring confirmation of positive results by another FDA approved method[52]. Zboromyrska *et al.* found that the sensitivity of ETEC ST/LT in xTAG GPP to be superior to their multiplex PCR used for routine testing[76].

Since xTAG GPP was made commercially available first through CE-IVD in May 2011 then FDA clearance first in January 2013 then extended approval in September 2014, there are a number of studies reported on xTAG GPP assay's clinical utility, overall performance and potential benefit in outbreak situation.

During the 2011 outbreak of a new aggressive enterohemorrhagic *E. coli* (EHEC) strain in Germany, in order to manage the exponential increase of suspected cases, xTAG GPP was used by Kliniken der Stadt Köln gGmbH, Cologne as a pre-screening tool partially because its high throughput (up to 96 samples per batch). More importantly, the assay discriminates Shiga-like toxin producing *E. coli* from a broad panel of pathogens that are implicated in infectious diarrhea, providing the dual benefit of rapid time-to-result and high throughput[78].

The reported clinical utility and overall performance of xTAG GPP varies from study to study, perhaps due to the inconsistency of sample population, sample types (e.g. fresh or frozen), sample processing and extraction methods used, and most importantly due to comparator methods used for the studies. In a study by Beckmann C, two study populations were used: 312 consecutive stool samples from 127 pediatric patients with gastroenteritis and 185 adult travelers suspected with parasitic infections. Multiplex xTAG GPP was evaluated against a combination of comparator methods: direct antigen detection (DAD), bacterial culture and microscopy. The study showed that rotavirus (27%) was the most prevalent in pediatric population while in adult traveler enterotoxigenic Escherichia coli (4 %) was the predominant pathogen identified by xTAG GPP. However, microscopic

examination reported a 23% *Blastocystis hominis* in adult travelers which is not covered by the xTAG GPP. All positive calls by xTAG GPP for Adenovirus, Rotavirus, *Clostridium difficile* and *Cryptosporidium* were confirmed, but not all positive calls for Norovirus and *Giardia* were confirmed[77]. In the study reported by Claas EC et al. the Nororvirus performance of xTAG GPP is comparable to real-time PCR, with a 100% sensitivity and specificity for Norovirus GI and 92.5% sensitivity and 97.6% specificity for Norovirus GII. Giardia was reported to be detected with 100% sensitivity and 98.9% specificity when using real-time PCR as comparator[79]. Similarly, in the same study, the positive agreement for Adenovirus 40/41 changed from 20% (4/20) when using real-time PCR as comparator but 100% (9/9) using bidirectional sequencing.

The sensitivity of xTAG GPP in detecting bacterial pathogens is overall comparable to bacterial culture. Worth noting is the different results from different studies for *Salmonella*. Compared to culture, *Salmonella* sensitivity was reported with 100% sensitivity (10/10) and 98.4% specificity (1143/1161) in the xTAG GPP FDA study, similarly 100% sensitivity (11/11) and 97.4% specificity (1349/1385) observed for *Salmonella* from study by Halligan et al[80]. However in the study published by Mengelle et al, the sensitivity of *Salmonella* was 77.8% (7/9) and specificity was 96.2% (356/370)[74].

BioFires FilmArray GI Panel—BioFires FilmArray GI Panel runs on the company's automated FilmArray instrument. The US-IVD version of the FilmArray GI assay simultaneously detects the nucleic acid from 13 bacterial, 5 viral and 4 parasites responsible for causing gastroenteritis in about 1 hour (Table 3)[53]. The FilmArray GI assay is completely automated with nucleic acid extraction, amplification and detection all occurring within the assay pouch. The hands-on preparation takes approximately 5 minutes and involves adding rehydration solutions to the assay pouch and loading the Cary-Blair stool sample which is achieved with a provided consumable[81]. Once the sample is loaded the FilmArray instrument subjects the sample to bead beating followed by nucleic acid extraction. The extracted nucleic acid is then amplified in a nested multiplex RT-PCR reaction to enrich the target sequences; after the initial PCR step is complete the amplified material is moved to the second PCR step which occurs on the film array where several singleplex PCR reactions occur. Detection of analytes is achieved by using endpoint melt curve analysis. The FilmArray GI assay returns one of four result calls: Detected, Not Detected, N/A and Invalid. The N/A result occurs when E. coli O157 is detected but stx1/stx2 is not detected, or when EPEC is detected with stx1/stx2. The Invalid results can be caused by instrument or software failure or internal control failure[53].

The result from the clinical trial with 1556 patients was that the Sensitivity of the FilmArray ranged from 94.5% – 100% and the specificity ranged from 97.1% – 100% depending on the target[53]. The overall assay success rate for samples in the prospective clinical trial was 99.4% for the initial testing and 99.9% upon repeat testing. It is unclear if this success rate includes invalid results due to PCR inhibition.

Care must be taken when comparing the published performance characteristics (sensitivity and specificity) of syndromic panels. Very often different comparator methods have been used to establish the assays performance characteristics. For example the microscopy was

used for establishing the performance of the parasitic targets in the xTAG GPP assay while a Nucleic Acid Amplification Test (NAAT) (bi-directional sequencing) was used to establish the performance of the same targets in the FilmArray GI assay. Although the NAAT's used were independent of the assay they are still molecular based assays which may provide a higher degree of concordance to a molecular test than microscopy would.

To date there is only one independent cohort study published using BioFire FilmArray GI panel. The study compares the performance of the Biofire FilmArray GI assay and the Luminex xTAG GPP assay[81]. The study included 230 prospectively collected stool samples in Cary-Blair medium and retrospective testing of 270 stool samples in Cary Blair medium. The IUO version of the BioFire assay was used, while the RUO version of the xTAG GPP assay was used. The IUO/RUO versions of these assays used in this study differ from the IVD version in that the IVD FilmArray GI does not claim for *Aeromonas* and the xTAG GPP IVD does not have a claim for *Yersinia enterocolitica*. Khare et al. found that both assays had high sensitivity and specificity (>90%) for the targets included in the IVD versions of the assays; see Table 4 for a summary of these results. In the prospective arm of this study the sensitivity of the BioFire and Luminex assay were nearly equivalent, *C. difficile* and Norovirus were the only two targets withdifferences in sensitivity between the assays. BioFire was more sensitive for *C. difficile* (100% vs. 95.8% for GPP) while Luminex was more sensitive for Norovirus (100% vs. 91.7%). In the retrospective arm of the study BioFire's sensitivity was higher than Luminex's. Khare et al. noted that this difference in sensitivity may be related to using only 100 μL of Cary-Blair stool for the extraction which is four fold less than what is used for xTAG GPP.

The specificity of xTAG GPP assay was nearly identical (within 1%) to the FilmArray GI panel. Norovirus GI/GII was the only target in the xTAG GPP assay that showed a significant difference in specificity (GPP: 88.3%, FilmArray: 99.7%). When Khare *et. al.* investigated this discrepancy it was found that the specificity problem may have been linked to a specific reagent lot, since upon retesting with a new lot of reagents the xTAG GPP specificity for Norovirus GII was 99.5%.

In terms of workflow and turn-around-time Khare *et. al.* found the FilmArray GI assay required approximately 5 minutes hands-on-time and a time to result of about 1 hour, while the xTAG GPP assay required approximately 60 minutes of hands-on-time and had a time to result of 5–6 hours. The throughput that could be obtained in a normal 8-hour shift with the FilmArray GI assay was 7–8 samples while the xTAG GPP assay could produce results for 96 samples within one shift[81].

Clinical relevance of the multiplex assays

Development and implementation of molecular techniques, especially those in multiplex formats have significantly improved workflow and diagnostic output in diagnosis of GI infections. However, clinical utility of multiplex assays is still to be further established. Earlier adopters and studies of these multiplex assays have indicated that multiplex assays could save time to detect a specific infectious organism, This is an important advantage because specific therapy could be initiated in case of bacterial infection. The clinical

relevance of a multiplex assay also lies in its negative predictive value. A negative result could mean de-isolate a patient and save the unnecessary burden otherwise. In a short communication by Kahlau P et al, it showed that the xTAG GPP assay provided same day results while conventional methods took about 3 days. Multiplex assays also gave 19 (of 104 total) positive results that were not requested by ordering physicians[82].

The performance characteristics and limitations of the multiplex molecular tests must be clearly understood by both laboratory personnel and clinicians to ensure proper utilization and interpretation. As techniques continue to advance, more and more microorganisms can be detected simultaneously from fecal specimens. Whether it is due to the microbial contamination, colonization, infection or disease merits further investigation. For example, a study performed by Navidad JF et al of using a 19-plex lab developed test demonstrated that a multiplex assay could be suitable as a primary screening tool for enteric bacteria, viruses, and parasites[41]. Additional extensive studies are needed to further investigate the clinical relevance of multiplex molecular assays in the diagnosis of GI infections. Prompt exchange of relevant information between the clinician and the laboratory is essential for the reliable molecular diagnosis of infectious gastroenteritis.

Self Assessment

1. An investigator has designed one primer set that targets the conserved regions of the bacterial 16S rRNA gene to detect a panel of common bacterial pathogens. This is considered as:

- **A.** Real-time PCR
- **B.** Broad-range PCR
- **C.** Multiplex PCR
- **D.** Random PCR
- **E.** Nested PCR

Correct answer is B. Broad-range PCR is different from the multiplex PCR described in the paper which involves the use of multiple sets of primers to target several loci.

2 Which of the following microbial agents is least likely to be a cause of infectious gastroenteritis?

- **A.** E. coli
- **B.** Rotovirus
- **C.** Salmonella
- **D.** Campylobacter
- **E.** E. histolytica

Correct answer is A. Non-pathogenic *E. coli* in gut is part of normal flora and should not be considered as a pathogen causing diarrhea. None of the other organisms are considered to be normal flora.

3 Which of the following pathogens requires an additional sample pre-processing step, such as bead-beating, prior to routine nucleic acid extraction?

A. *Campylobacter* ssp.

B. Yersinia enterocolitica

C. Norovirus

D. Clostridium difficile

E. *Cryptosporidium* spp.

Correct answer is E. Bead-beating helps to crack open lysis resistant parasitic cyst (such as *cryptosporidium* oocysts) to ensure the efficiency of the subsequent NA extraction. This applies to *Giardia* as well.

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Key points

1. Conventional laboratory diagnostic techniques are time consuming and often lack sensitivity and specificity in detecting the causes of infectious gastroenteritis.
2. Multiplex PCR based tests have made their way into the gastroenterology diagnostic arena in recent years due to their high sensitivities and specificities, as well as their capacity of “one stone for many birds”.
3. Numerous laboratory-developed multiplex PCR tests have been reported for detection and identification of microbial pathogens in stool and, recently, two FDA cleared multiplex tests, the Luminex xTAG GPP and Biofire FimArray GI test, have been quickly applied in clinical practice.
4. The clinical relevance of the multiplex PCR tests is to be further determined in the diagnosis of infectious gastroenteritis.

Table 1

Recently published multiplex research assays for the syndromic identification of gastroenteritis causing agents (data from Refs 37, 41–48)

Research Assay Development Site/Institute/Laboratory	Pathogen targeted in the multiplex assay	Sample Type and Nucleic acid Amplification / Detection Method	Performance	References
Milwaukee Health Department Laboratory	Total 19 targets: *Campylobacter jejuni, Salmonella, Shigella, ETEC STEC, E. coli O157:H7, Vibrio cholerae, Yersinia enterocolitica, and toxigenic Clostridium difficile) Giardia lamblia, Cryptosporidium, Entamoeba histolytica*, Norovirus GI/ GII, Adenovirus 40/41 and Rotavirus A	Stool, multiplex RT-PCR – Luminex microsphere detection	Sensitivity: 94.5% (90% - 97%) Specificity: 99%	Navidad JF, 2013 [41]
Division of Infectious Diseases and International Health, University of Virginia, US	Total 15 pathogens: Adenovirus, Astrovirus, Norovirus GII, Rotavirus, and Sapovirus, *Campylobacter, Salmonella, Vibrio cholerae*, EAEC, ETEC, EPEC, ETEC, *Cryptosporidium, Giardia lamblia*, and *Entamoeba histolytica*	multiplex RT-PCR-Luminex microsphere detection	Sensitivity: 86.2% (median) Specificity: ≥ 95%.	Liu J, 2014 [37]
Department of Pediatrics, McMaster University, Hamilton, ON, Canada	Total 16 pathogens: EHEC (stx1/stx2), *E. coli* O157, *Salmonella Shigella, Campylobacter, Yersinia entercolitica Clostridium difficile, Clostridium difficile tcd* B, *Listeria monocytogenes, Vibrio parahaemolyticus* Norovirus GI/GII, Rotaviruses, Astroviruses, Adenoviruses 40/41, Sapoviruse, *Giar dia lamblia and Cryptosporidium*	Nanolitre real-time PCR panel	*Cryptosporidium* sensitivity greater than microscopy or enzyme immunoassay	Goldfarb DM, 2013 [42]
Department of Microbiology, Faculty of Medicine, Chiang Mai University, Chiang Mai, Thailand	Total 10 viruses: Rotavirus (A/C), Adenovirus, Norovirus GI/ GII, Sapovirus, Astrovirus, Aichi virus, Parechovirus, and Enterovirus	multiplex RT-PCR	47.2% positivity rate on infants and children with acute gastroenteritis	Khamrin P 2011 [43]
Provincial Laboratory for Public Health, Edmonton, Alberta, Canada	Total 5 viruses: Rotavirus, Norovirus, Sapovirus, Astrovirus, and Adenovirus	Stool, real-time PCR	Higher positivity rate than with microscopy	Pang XL 2014 [44]
Regional Virus Laboratory, Royal Victoria Hospital, Belfast, Northern Ireland, United Kingdom	Total 4 viruses; Rotavirus, Norovirus, Astrovirus, and Adenoviruses 40/41	Stool, real-time (TaqMan)	Sensitivity: 95% - 99% Specificity: 95% - 100%	Feeney SA 2011 [45]
Department of Microbiology and Immunology, Weill Medical College of Cornell University	Total 7 bacteria: *Campylobacter, Vibrio, Shigella, Salmonella Listeria monocytogenes, Yersinia enterocolitica and diarrheagenic Escherichia coli.*	Stool, multiplex PCR/ligation detection reaction (LDR) assay	Sensitivity: 91% - 100% Specificity: 98% - 100%	Rundell MS, 2014 46

Research Assay Development Site/Institute/Laboratory	**Pathogen targeted in the multiplex assay**	**Sample Type and Nucleic acid Amplification / Detection Method**	**Performance**	**References**
Laboratory Medical Science Cluster, Universiti Teknologi MARA (UiTM), Malaysia	Total 4 bacteria: *Salmonella, Shigella*, EHEC and *Campylobacter*	Stool, multiplex PCR	Contrived sample Sensitivity and Specificity: 100%. LoD 1×10^3 CFU/mL	Al-Talib H 2014 [47]
Department of Biology, Faculty of Science, Mahidol University, Bangkok 10400, Thailand	Total 4 toxin genes *of Clostridium difficile* tcdA, tcdB, cdtA, and cdtB	Stool multiplex PCR	Sensitivity and Specificity: 100%. LoD: ~ 22 copies	Chankhamhaengdecha S, 2013 [48]

Table 2

Commercial Multiplex assays available for syndromic identification of gastroenteritis causing agents

Manufacture – Test Name	US-IVD	CE-IVD	Number Pathogens Detected	Time to Result (hours)	Throughput	Sample pre-processing	Stool Source	Technology	Specialized equipment required	Complexity
Luminex - xTAG GPP	Yes	Yes	14	5	96	Yes	Raw, Cary-Blair	Multiplex endpoint RT-PCR, Hybridization	Yes	High
BioFire – FilmArray GI	Yes	No	22	1	1	No	Cary-Blair	Multiplex RT-PCR, melt analysis	Yes	Low
BD MAX - Enteric bacterial Panel	Yes	Yes	4	4	24	No	Raw, Cary-Blair	Microfluidic real-time PCR	Yes	Low
	No	Yes	2	4	24	No	Raw	Microfluidic real-time PCR	Yes	Low
BD MAX – Enteric Virus BD MAX – Enteric Parasite	No	Yes	3	4	24	No	Raw, formalin-fixed	Microfluidic real-time PCR	Yes	Low
Nanosphere - Verigene Enteric Pathogen	Yes	Yes	8	2	1	No	Cary-Blair	Microarray	Yes	Low
Hologic (Gen-Probe) - ProGastro SSCS	Yes	Yes	4**	4	96†	Yes	Cary-Blair	Multiplex Realtime PCR	No	Medium
PathoFinder - Gastrofinder Smart 17 Fast	No	Yes	17	5	96	Yes	Not specified	Multiplex RT-PCR, ligation probe melt analysis	No	High
r-Biopharm - Rida Gene - Hospital stool (HS), Bacterial Stool (BS) Viral Stool (VS), Parasitic Stool (PS)	No	Yes	HS=3 BS=3 VS=4 PS=4	2	96†	Yes	Raw	Multiplex Realtime PCR	No	Medium
Seegene - SeeplexDiarrhea ACE - Viral (V), Bacterial 1 (B1) and 2 (B2)	No	Yes	V=4 B1=5 B2=5	9–10	96†	Yes	Raw, Cary-Blair, Rectal swabs	Multiplex PCR, capillary electrophoresis	No	High
Serosep - EntericBio Gastro Panel 1 (P1) and 2 (P2)	No	Yes	P1=4, P2=6	3	96†	No	Not specified	Multiplex Realtime PCR	No	Low

Manufacture – Test Name	US-IVD	CE-IVD	Number Pathogens Detected	Time to Result (hours)	Throughput	Sample pre-processing	Stool Source	Technology	Specialized equipment required	Complexity
Fast-Track Diagnostics - FTD Stool Parasites (P), EPA, Bacterial (B), Viral (V)	No	Yes	P=3 EPA=3 B=6 ** V=5 **	6	96 B=48 V=48	Yes	Not specified	Multiplex Realtime PCR, RT-PCR	No	Medium
Diagenode- G-DiaBact (B), G-DiaNota (V), G-Diapara (P)	No	Yes	B=2 V=3 P=3	4	96	yes	Raw	Multiplex Realtime PCR, RT-PCR	No	Medium
Genetic Signatures - EasyScreen Enteric Bacteria (B), Viral (V), Protozoan (P)	No	Yes	B=9 *** V=8 *** P=5 **	5	B=32 V=32 P=48	Yes	Not specified	Multiplex Realtime PCR, RT-PCR	No	Medium
AusDiagnostics - Faecal Bacteria (B), GI Parasites (P)	No	No	B=5 P=4	3	B=12 P=14	Yes	Not specified	Multiplex Tandem Real-Time RT-PCT	Yes	Low
Genomica - CLART EnteroBac	No	Yes	7 **	5	48	Yes	Raw	Multiplex PCR, micro-array	Yes	High

†When running one panel

**Two tubes/wells required for coverage

***Three tubes/well required for coverage

Table 3

Gastroenteritis causing pathogens detected by commercially available syndromic multiplex assays

Manufacture/Assay	*Aeromonas*	*Campylobacter*	*Clostridium difficile*	*Clostridium perfringens*	Enteroaggregative *E. coli* (EAEC)	Enteropathogenic *E. coli* (EPEC)	Enterotoxigenic *E. coli* (ETEC) *lt/st*	Shiga toxin *E. coli* (STEC) *stx1/stx2*	*E. coli* O157	Listeria monocytogenes	*Plesiomonas shigelloides*	*Salmonella*	*Shigella*	*Vibrio*	*Vibrio cholerae*	*Yersinia enterocolitica*	Adenovirus F40/41	Astrovirus	Enterovirus	Norovirus GI/GII	Rotavirus A	Sapovirus	*Cryptosporidium*	*Blastocystis hominis*	Dientamoba fragilis	*Cyclospora cayetanensis*	*Entamoeba histolytica*	*Giardia lamblia*
Luminex - xTAG GPP		✓	✓				✓	✓	✓			✓	✓		✓	✓*	✓			✓	✓		✓				✓	✓
BioFire – FilmArray GI	✓*	✓	✓		✓	✓	✓	✓	✓		✓	✓	✓	✓	✓	✓	✓	✓		✓	✓	✓	✓			✓	✓	✓
BD MAX - Enteric bacterial (B), Virus* (V), Parasite* (P)		✓ B						✓ B				✓ B	✓ B							✓ V	✓ V		✓ P				✓ P	✓ P
Nanosphere - Verigene Enteric Pathogen		✓						✓				✓	✓	✓		✓				✓	✓							
Hologic (Gen-Probe) - ProGastro SSCS		✓						✓				✓	✓															
PathoFinder - Gastrofinder Smart 17 Fast	✓	✓	✓				✓	✓	✓			✓	✓			✓	✓	✓		✓	✓		✓		✓		✓	✓
r-Biopharm - Rida Gene - Hospital stool (HS), Bacterial Stool (BS) Viral Stool (VS), Parasitic Stool (PS)		✓ B	✓ HS									✓ B				✓ B	✓ V2	✓ V2		✓ V2	✓ V2		✓ P		✓ P		✓ P	✓ P
Seegene - SeeplexDiarrhea ACE - Viral (V), Bacterial 1 (Ba) and 2 (Bb)	✓ Bb	✓ Ba	✓ Ba	✓ Bb				✓ Bb	✓ Bb			✓ Ba	✓ Ba	✓ Ba		✓ Ba	✓ V	✓ V		✓ V	✓ V							
Serosep - EntericBio Gastro Panel 1 (P1) and 2 (P2)		✓						✓				✓	✓										✓					✓
Fast-Track Diagnostics - FTD Stool Parasites (P), EPA, Bacterial (B), Viral (V)		✓ B2	✓ B2					✓ B2				✓ B2	✓ B2			✓ B2	✓ V3	✓ V3		✓ V3	✓ V3	✓ V3	✓ P				✓ P	✓ P
Diagenode- G-DiaBact (B), G-DiaNota (V), G-Diapara (P)		✓ B										✓B											✓ P				✓ P	✓ P
Genetic Signatures - EasyScreen Enteric Bacteria (B), Viral (V), Protozoan (P)	✓ P3	✓ P3	✓ P3					✓ P3		✓ P3		✓ P3	✓ P3	✓ P3		✓ P3	✓ V3	✓ V3	✓ V3	✓ V3	✓ V3		✓ P2	✓ P2	✓ P2		✓ P2	✓ P2
AusDiagnostics - Faecal Bacteria (B), GI Parasites (P)		✓ B	✓ B									✓ B	✓ B										✓ P		✓ P		✓ P	✓ P
Genomica – CLART EnteroBac	✓	✓	✓		← undifferentiated →							✓	✓			✓												

Numbers indicates the number of tubes/wells required for each test or sub-panel

*Test/analyte not included in IVD version

Table 4

A summary of Khare et al. results for the sensitivity and specificity of the Biofire FilmArray GI assay (IUO) and the Luminex xTAG GPP assay (RUO) in a 230 sample prospective and 270 sample retrospective study

	Prospective				Retrospective				Combined			
	FilmArray		xTAG GPP		FilmArray		xTAG GPP		FilmArray		xTAG GPP	
Target	Sens.	Spec.	Sens.†	Spec.†	Sens.	Spec.	Sens.†	Spec.†	Sens.	Spec.	Sens.†	Spec.†
Aeromonas spp.	100%	100%	-	-	23.8%	100%	-	-	27.3%	100%	-	-
Campylobacter	100%	100%	100%	100%	96.6%	99.6%	79.3%	100%	96.9%	99.8%	81.3%	100%
C. difficile toxin A/B	100%	96.6%	95.8%	97.2%	91.7%	97.9%	91.7%	98.3%	95.0%	97.3%	93.3%	99.1%
Plesiomonas shigelloides	ND	100%	-	-	100%	100%	-	-	100%	100%	-	-
Salmonella spp.	100%	99.6%	100%	100%	100%	100%	83.3%	100%	100%	99.8%	84.0%	100%
Yersinia enterocolitica	ND	100%	ND	100%	100%	99.6%	48.1%	100%	100%	99.8%	48.1%	100%
Vibrio spp.	ND	100%	-	-	ND	ND	-	-	ND	100%	-	-
Vibrio cholerae	ND	100%	ND	100%	ND	ND	ND	ND	ND	100%	ND	100%
EAEC	ND	ND	-	-	ND	ND	-	-	ND	100%	-	-
EPEC	ND	ND	-	-	ND	ND	-	-	ND	100%	-	-
ETEC	100%	100%	100%	100%	ND	ND	ND	ND	100%	100%	100%	100%
STEC	ND	100%	ND	100%	100%	99.2%	96.4%	99.6%	100%	99.4%	96.4%	99.8%
E. coli 0157	ND	100%	ND	100%	100%	100%	90.9%	99.6%	100%	100%	90.9%	99.8%
Shigella/EIEC	100%	99.6%	100%	99.5%	90.9%	99.6%	81.8%	ND	92.3%	99.6%	84.6%	100%
Cryptosporidium	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%

	Prospective				Retrospective				Combined			
	FilmArray		xTAG GPP		FilmArray		xTAG GPP		FilmArray		xTAG GPP	
Target	Sens.	Spec.	Sens.†	Spec.†	Sens.	Spec.	Sens.†	Spec.†	Sens.	Spec.	Sens.†	Spec.†
Cyclospora cayetanensis	ND	100%	-	-	ND	ND	-	-	ND	100%	-	-
Entamoeba histolytica	ND	100%	ND	100%	0%	100%	100%	100%	0.0%	100%	100%	100%
Giardia lamblia	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%
Adenovirus 40/41	100%	99.2%	100%	100%	90%	99.2%	80%	100%	90.9%	99.4%	81.8%	100%
Norovirus GI/GII	91.7%	99.5%	100%	90.8%	93.2%	100%	93.2%	85.8% / 99.5%*	92.9%	99.6%	94.6%	88.3%/95.3%*
Rotavirus A	ND	99.6%	ND	99.1%	100%	99.6%	92.9%	99.6%	100%	99.2%	92.9%	99.4%
Sapovirus	100%	ND	-	-	90.3%	100%	-	-	93.2%	100%	-	-
Astrovirus	100%	ND	-	-	100%	100%	-	-	100%	100%	-	-

Sens.: Sensitivity, Spec.: Specificity

† 100 μL Cary-Blair stool input used; Luminex recommends 400 μL of Cary-Blair stool in the US-IVD version of the xTAG GPP package Insert

* Retrospective false positives for Norovirus GII were re-tested with a new lot of xTAG GPP; 31 of 32 false positives were resolved with retesting.

Data from Khare R, Espy MJ, Cebelinski E, et al. Comparative evaluation of two commercial multiplex panels for detection of gastrointestinal pathogens by use of clinical stool specimens. J Clin Microbiol. 2014;52(10):3667–3673.