

Evaluation of the Luminex xTAG Gastrointestinal Pathogen Panel and the Savyon Diagnostics Gastrointestinal Infection Panel for the detection of enteric pathogens in clinical samples

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Infectious gastrointestinal disease is caused by a diverse array of pathogens, and is a challenging syndrome to correctly diagnose and manage. Conventional laboratory diagnostic methods are often time-consuming and frequently suffer from low detection rates. Two commercial multiplex nucleic acid amplification tests [Luminex xTAG Gastrointestinal Pathogen Panel (GPP) and Savyon Diagnostics Gastrointestinal Infection Panel (GIP)] were applied to 1000 stored diarrhoeal clinical stool samples. The Luminex xTAG GPP and Savyon GIP detected *Campylobacter* in 42/44 and 44/44 culture-positive samples, *Salmonella* in 4/4 and 3/4 culture-positive samples, *Shigella* in 1/1 culture-positive sample, *Clostridium difficile* toxin in 32/35 ELISA-positive samples, and *Giardia* in 6/6 wet-preparation-microscopy-positive samples, respectively. When the Luminex GPP assay was used concurrently with conventional methods for 472 clinical samples, it detected *Campylobacter* in 22/22 culture-positive samples, *Salmonella* in 1/1 culture-positive sample, *Clostridium difficile* toxin in 14/14 ELISA-positive samples and *Giardia* in 4/4 wet-preparation-microscopy-positive samples. The pathogen/toxin detection rate for conventional methods in both sample sets was <10%. The Luminex xTAG GPP detection rate was 24.8% in the stored samples and 32.6% in the concurrently tested samples. The Savyon GIP detection rate was 22.5%. From stored samples, 2.4% of Luminex xTAG GPP detections and 3.1% of Savyon GIP detections could not be confirmed using alternative nucleic acid amplification tests. Enhanced detection rates resulted from increased detection of pathogens routinely sought using conventional methods and were also due to ascertainment of micro-organisms that current testing strategies do not diagnose. Use of multiplex nucleic acid amplification tests will allow clinical laboratories to diagnose infectious gastroenteritis in more patients with diarrhoeal disease by increasing the sensitivity of pathogen detection and by reducing the selective bias of current strategies. The clinical and economic impact of these results warrants further investigation.

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INTRODUCTION

Infectious gastrointestinal illness is a clinical syndrome whose aetiology is as varied as its presentation. Symptoms range from mild, self-limiting diarrhoea to potentially life-threatening haemolytic uraemic syndrome or pseudomembranous colitis. Attempts to estimate the human and economic cost of infectious intestinal diseases (IIDs) have demonstrated their far-reaching effects within hospitals and in the wider community. Increased sensitivity and rapidity of result reporting have the potential to aid infection control efforts, reduce overall social and healthcare

costs, and improve treatment outcomes (Hoffmann *et al.*, 2012; Lopman *et al.*, 2004; Roberts *et al.*, 2000; STEC Workshop Reporting Group, 2012; Vonberg *et al.*, 2008).

Many conventional techniques for the microbiological diagnosis of IID (e.g. microscopy, culture and ELISA) are labour-intensive and time-consuming, and frequently result in low detection rates. A plethora of in-house and commercial nucleic acid amplification tests (NAATs) have been developed to detect a wide range of individual micro-organisms associated with diarrhoeal illness. Many authors have also reported the successful use of multiplexed assays that target groups of bacteria, parasites and viruses (Coupland *et al.*, 2013; Cunningham *et al.*, 2010; de Boer *et al.*, 2010; Higgins *et al.*, 2011; Koziel *et al.*, 2013; McAuliffe *et al.*, 2013; Schuurmann *et al.*, 2007; Stark *et al.*, 2011). Several publications show the feasibility and accuracy of identifying bacteria, parasites and viruses within a single

Abbreviations: ETEC, enterotoxigenic *Escherichia coli*; GIP, Gastrointestinal Infection Panel; GPP, Gastrointestinal Pathogen Panel; IID, infectious intestinal disease; MFI, median fluorescence intensity; NAAT, nucleic acid amplification test; STEC, shiga-like toxin-producing *Escherichia coli*.

test (Claas *et al.*, 2013; Kahlau *et al.*, 2013; Mengelle *et al.*, 2013).

The Luminex xTAG Gastrointestinal Pathogen Panel (GPP) and Savyon Diagnostics Gastrointestinal Infection Panel (GIP) are NAAT-based tests that allow for the detection of multiple infectious agents of intestinal disease in one test (Table 1). Both Luminex and Savyon assays employ conventional PCR followed by end-point detection methods. The former utilizes the xTAG universal sorting system for amplicon detection, whereas the latter is based on NanoChip electronic microarray analysis.

This paper reports on an assessment of the analytical capability of the GPP and GIP assays to identify gastrointestinal pathogens from stored clinical faecal specimens submitted for conventional testing. Concurrent use of the Luminex assay and a comparison with conventional test results are also presented. The potential benefits and limitations of molecular-based IID syndromic strategies are discussed.

METHODS

Samples. One thousand faecal samples (submitted by hospital and community doctors) were collected over the period of one summer month in 2012 for analysis using the Luminex GPP and Savyon GIP

assays. This was accomplished by inoculating residual sample material (100 µl aliquot or a pea-sized amount of all liquid and semi-formed diarrhoeal stool specimens where there was sufficient residual sample) into 0.9 ml NucliSENS easyMag lysis buffer (bioMérieux) at the same time that the samples were processed for conventional methods. Where there was insufficient residual material, only conventional testing was undertaken. Conventional testing was undertaken immediately, whilst samples in lysis buffer were then stored at room temperature prior to extraction and Luminex testing <4 weeks later. The same extracts used for the Luminex testing were subsequently stored at -80 °C for 9 months before Savyon testing was undertaken as the assay was still in development at the beginning of the study. In total, 472 liquid faecal samples from in-patient and out-patient settings were tested over a period of two winter months (December 2012–January 2013) concurrently using the Luminex GPP assay and conventional methods. To increase sensitivity in the Luminex assay, a 200 µl aliquot of sample was utilized. Conventional testing and lysis buffer inoculation were undertaken on the same day as receipt of sample. Samples in lysis buffer were tested within 24 h of inoculation Monday–Friday. Conventional testing and lysis buffer inoculation were performed daily at weekends. However, samples in lysis buffer received subsequent to testing on a Friday were stored at 4 °C until Monday morning when they were tested using the Luminex assay. Funding for Savyon testing did not allow for evaluation of the assay on the additional 472 samples tested concurrently using the Luminex GPP assay.

Conventional testing. Samples were cultured for *Campylobacter*, *Escherichia coli* O157, *Salmonella* and *Shigella* according to UK Standards for Microbiology Investigations (Health Protection Agency, 2013a). Microscopic examination for ova, cysts and parasites was also

Table 1. Targets detected using conventional diagnostic methods, Luminex GPP and Savyon GIP assays

√, Pathogen detectable; ×, pathogen not detectable.

Pathogen/target	Detection method		
	Conventional diagnostics	Luminex GPP	Savyon GIP
<i>Campylobacter</i>	√	√	√
<i>Clostridium difficile</i> toxin A/B	√*	√	√
<i>Escherichia coli</i> O157	√	√	×
Enterotoxigenic <i>Escherichia coli</i> (ETEC)	×	√	×
Shiga-like toxin-producing <i>Escherichia coli</i> (STEC)	×	√	×
<i>Salmonella</i>	√	√	√
<i>Shigella</i>	√	√	√
<i>Vibrio cholerae</i>	√*	√	×
<i>Yersinia enterocolitica</i>	√*	√	×
<i>Cryptosporidium</i>	√†	√	√
<i>Giardia lamblia</i>	√†	√	√
<i>Entamoeba histolytica</i>	√†	√	√
<i>Dientamoeba fragilis</i>	×	×	√
Adenovirus 40/41	×	√	×
Norovirus	×	√	×
Rotavirus A	×	√	×

*Conventional testing for *Clostridium difficile* toxins A/B was only performed on patients aged >2 years old within a hospital or care home, and although conventional testing for *V. cholerae* and *Y. enterocolitica* was available it was not undertaken due to the absence of clinical details suggestive of disease in the samples tested.

†Microscopic examination of stool samples allows for the detection of many more parasites than those listed above and detected using most molecular assays.

undertaken using the same Standards (Health Protection Agency, 2013b). Detection of *Clostridium difficile* toxins A and B was accomplished using a nationally accepted two-step algorithm (Department of Health, 2012) using enzyme immunoassay tests (Techlab) where a toxin ELISA test is performed only after a sample has tested positive using a GDH ELISA test. No routine testing for viruses was performed, although, where requested, an in-house norovirus assay and the VIKIA Rota-Adeno immunochromatographic test (bioMérieux) for the combined detection of rotavirus and adenovirus were available.

Molecular assay specimen pre-treatment and nucleic acid extraction. Samples were prepared and extracted according to the Luminex manufacturer's instructions with the following alterations: to enhance recovery of parasite nucleic acid, bead beating was undertaken using a Mini-Beadbeater (BioSpec) as opposed to simple vortexing as recommended; total nucleic acid was extracted using the NucliSENS easyMag (bioMérieux) specific B protocol using 100 µl diluted (500 µl magnetic silica plus 550 µl elution buffer) magnetic silica as opposed to the specific A protocol as recommended.

Each extraction was controlled by the addition of known positive material (pooled positive clinical samples containing either rotavirus or norovirus in addition to *Campylobacter* and *Cryptosporidium*) and negative material (pooled clinical samples negative for all target pathogens).

Luminex GPP amplification and detection. All the reagents for amplification were provided in the xTAG GPP reagent kit (Luminex) as was bacteriophage MS2 internal control material that was spiked into each sample lysis buffer prior to extraction to control for the presence of PCR inhibitors. A 10 µl aliquot of chilled template (sample, positive or negative control) was added to individual aliquots of 15 µl PCR mix. PCRs were prepared according to the manufacturer's instructions ensuring that, once thawed, reagents and eluates were constantly chilled during PCR assay set-up. Amplification was accomplished using a pre-heated Veriti 96-well thermal cycler (Life Technologies) with the ramp rate reduced to 65%. PCR cycling conditions were in accordance with the manufacturer's instructions: one cycle at 53 °C for 20 min; one cycle at 95 °C for 15 min; 38 cycles of 95 °C for 30 s, 58 °C for 30 s and 72 °C for 30 s; followed by one cycle at 72 °C for 2 min. At the end of the PCR cycling the products could be kept at 4 °C for up to 12 h prior to hybridization and detection.

Hybridization and detection reagents were supplied as part of the xTAG GPP kit (CE-IVD) and these processes were carried out according to the manufacturer's instructions within 1 h of completion of the PCR process. Detection was undertaken using the GPP protocol on a MAGPIX instrument (Luminex) with TDAS v.1.11 GPP xTAG Data Analysis Software.

Savyon GIP amplification and detection. Savyon testing was accomplished using the 1000 stored (−80 °C) easyMag extracts. Amplification and detection of target and endogenous internal control were undertaken according to the Savyon GIP application note v.23.2.13 (Savyon Diagnostics, unpublished data) using the reagents supplied (Savyon Diagnostics). Each reaction included: 6 µl primer mix, 0.75 µl FastStart *Taq* DNA polymerase (Roche), 12.5 µl LS amplification buffer, 2.45 µl RNase-free water and 0.3 µl PCR enhancer. Once thawed, reagents and eluates were constantly chilled during assay set-up. A 3 µl aliquot of sample extract in addition to positive and negative PCR controls was added to individual aliquots of 22 µl PCR mix in a chilled 96-well PCR plate. The plate was then covered using a microseal that was not removed prior to detection, but pierced using the detection instrument's probe. Amplification was accomplished using a Veriti 96-well thermal cycler with the ramp rate at 100%. PCR cycling conditions were: one cycle at 95 °C for 4 min;

43 cycles of 95 °C for 30 s, 60 °C for 1 min and 72 °C for 45 s; followed by one cycle at 72 °C for 5 min. At the end of the PCR cycling the products were kept at 4 °C prior to detection for <1 h.

GIP detection reagents, capture/reporter and reference packs and buffers, high salt, low salt, target prep, and CAP_{down} sample buffers (Savyon Diagnostics) were brought to room temperature and mixed immediately prior to use. Fresh, filtered (0.2 µM filter) distilled water and wash solution (500 ml 50 mM histidine solution and 2.5 ml 0.1% Triton X-100 solution) were loaded onto the NanoChip 400 instrument (Savyon Diagnostics) for each run. A NanoChip cartridge was kept at room temperature for 15 min before insertion, initialization and priming on the NanoChip instrument. Reagents and PCR products were placed into the relevant positions, and were run on the NanoChip 400 instrument before results were exported and analysed by Savyon.

Pathogens detected by each conventional and commercial test are listed in Table 1.

Alternative molecular assays. Molecular assays other than the Luminex GPP and Savyon GIP were used to investigate discrepant results or confirm results for pathogens not detected by conventional methods or GPP and GIP from the stored sample cohort. These assays included: proprietary Luminex validated conventional PCR assays for *Clostridium difficile* toxins A/B, *Escherichia coli* O157, other enterotoxigenic *Escherichia coli* (ETEC) and shiga-like toxin-producing *Escherichia coli* (STEC), and rotavirus A (targeting sequences distinct from those used in the xTAG GPP assay); Diagenode PCR assays for *Campylobacter*, *Clostridium difficile*, *Entamoeba histolytica* and *Dientamoeba fragilis*; laboratory-developed real-time assays for norovirus and adenovirus; and previously published *Yersinia enterocolitica* (Lambertz *et al.*, 2008) and *Giardia lamblia* (Verweij *et al.*, 2004) real-time assays. Of the samples that were tested concurrently using the Luminex assay, only *Giardia*-positive samples that were not detected by conventional means were tested using an alternative method (Verweij *et al.*, 2004) due to results from the stored sample testing having shown other targets to be detected reliably by the assay.

RESULTS

Stored samples

Of the 1000 faecal samples tested using the GPP and GIP assays: 995 were processed for ova, cysts and parasites, 991 were cultured for *Campylobacter*, *Escherichia coli* O157, *Salmonella* and *Shigella*, and 597 were tested for the presence of *Clostridium difficile* toxins A/B.

Of the 1000 stored samples tested: 90 were positive using conventional methods, 225 with the Savyon GIP assay and 248 with the Luminex GPP assay. The molecular assays demonstrated equivalent overall confirmed detection rates for pathogens common to both tests that were routinely sought after using conventional techniques.

Table 2 illustrates the breakdown of all pathogens detected by assay type. The Luminex GPP assay detected all pathogens identified using routine tests with the exception of two *Campylobacter* culture-positive samples and three *Clostridium difficile* toxin A/B ELISA-positive samples. The Savyon GIP assay did not detect one *Salmonella* culture-positive sample and the same three *Clostridium difficile*

Table 2. Pathogen detection in 1000 stored samples by assay type

Figures in parentheses correspond to the number of samples positive using conventional methods that were also detected using the Luminex and Savyon assays. Discrepant specimens are defined as those detected by either Luminex or Savyon assays that were negative or not tested by routine methods and that could not be confirmed using alternative molecular tests.

Pathogen/target	No. detected			No. of discrepant specimens
	Conventional diagnostics	Luminex GPP	Savyon GIP	
<i>Campylobacter</i>	44	74 (42)	82 (44)	5*
<i>Clostridium difficile</i> toxin A/B	35	91 (32)	84 (32)	1*
<i>Escherichia coli</i> O157	0	2	–	–
ETEC	–	14	–	–
STEC	–	5	–	–
<i>Salmonella</i>	4	7 (4)	4 (3)	2†
<i>Shigella</i>	1	2 (1)	2 (1)	–
<i>Vibrio cholerae</i>	–	0	–	–
<i>Yersinia enterocolitica</i>	–	2	–	–
<i>Cryptosporidium</i>	0	0	0	–
<i>Giardia lamblia</i>	6	10 (6)	8 (6)	2†
<i>Entamoeba histolytica</i>	0	1	0	1†
<i>Dientamoeba fragilis</i>	–	–	45	1*
Adenovirus 40/41	–	4	–	–
Norovirus	–	25	–	–
Rotavirus A	–	11	–	1†
Total	90	248	225	–

*Savyon discrepancy.

†Luminex discrepancy.

toxin A/B ELISA-positive samples as the Luminex GPP assay. The Luminex and Savyon assays showed 88 % agreement for pathogens common to both tests (*Campylobacter*, *Clostridium difficile* toxins A/B, *Salmonella*, *Shigella*, *Entamoeba histolytica* and *Giardia*). Luminex GPP assay positives for pathogens not detected by the Savyon GIP assay (toxigenic *Escherichia coli*, *Y. enterocolitica*, norovirus, rotavirus A and adenovirus) were confirmed as true positives using alternative molecular assays in all but one case, where one of the 11 rotavirus A-positive samples could not be confirmed. Only one of the six Savyon GIP *Campylobacter* detections not detected using the Luminex GPP assay was confirmed using an alternative molecular method. The solitary Savyon GIP-only *Clostridium difficile* toxin A/B detection was also not confirmed. Of the 45 *D. fragilis*-positive samples, 44 were confirmed using an alternative molecular method.

The enhanced detection of *Campylobacter* and *Clostridium difficile* toxins A/B accounted for ~50 % of the increased detection of the molecular assays, although more samples with *Salmonella*, *Shigella*, *Escherichia coli* O157 and *Giardia* were also seen using these tests. Approximately 27 % of the increased detection rate for the Luminex GPP assay originated from the detection of micro-organisms that were not tested for using routine strategies (*Y. enterocolitica*, non-O157 ETEC/STEC, norovirus, rotavirus A and adenovirus 40/41). Nearly 30 % of the increased detection

rate of the Savyon GIP assay was due to detection of *D. fragilis*, which was also not identifiable using the conventional techniques currently employed for routine faecal diagnostics or the Luminex GPP assay.

In total, five samples were positive for multiple targets using the Savyon GIP assay compared with ten samples with Luminex testing (Table 3).

Concurrently tested samples

Of the 472 faecal samples tested concurrently using the Luminex GPP assay: 453 were processed for ova, cysts and parasites, and were cultured for *Campylobacter*, *Escherichia coli* O157, *Salmonella* and *Shigella*, and 373 were tested for the presence of *Clostridium difficile* toxins. As a result of several positive samples having been missed in stored sample testing, 200 µl stool specimen was used for testing samples concurrently with conventional methods to enhance Luminex GPP assay sensitivity.

With concurrent testing, the Luminex GPP assay was able to detect all of the pathogens identified using conventional methods (Table 4). Luminex GPP detected an additional 114 pathogens, only 46 of which were detectable with the conventional methods employed, including: *Campylobacter* ($n=12$), *Clostridium difficile* toxin A/B ($n=26$), ETEC/STEC ($n=9$), *Shigella* ($n=2$), *Giardia* ($n=6$), adenovirus 40/41 ($n=3$), norovirus ($n=55$) and rotavirus A ($n=1$). Six

Table 3. Mixed infections/colonizations detected in 1000 stored samples using molecular assays for enteric pathogen detection

Assay	Co-pathogens detected (n)
Savyon GIP	<i>Shigella</i> and <i>D. fragilis</i> (1) <i>Clostridium difficile</i> toxin A/B and <i>Campylobacter jejuni</i> (1) <i>Campylobacter jejuni</i> and <i>D. fragilis</i> (1) <i>Giardia</i> and <i>D. fragilis</i> (2)
Luminex GPP	<i>Clostridium difficile</i> toxin A/B and <i>Campylobacter</i> (1) <i>Clostridium difficile</i> toxin A/B and STEC (1) <i>Clostridium difficile</i> toxin A/B and norovirus (2) Norovirus and <i>Salmonella</i> (1) Norovirus and rotavirus (2) Norovirus, rotavirus and <i>Clostridium difficile</i> toxin A/B (1) Norovirus and <i>Yersinia enterocolitica</i> (1) Adenovirus and ETEC (1)

co-infections were identified by Luminex GPP (four norovirus with *Clostridium difficile* toxin A/B, one norovirus with *Shigella*, and one sample with *Giardia*, *Shigella* and *Clostridium difficile* toxin A/B). Nine of the 10 *Giardia* positives were confirmed using an alternative NAAT.

DISCUSSION AND CONCLUSIONS

Conventional detection of *Campylobacter* species is documented as achieving 65–80% sensitivity when compared with confirmed molecular results (Bessède *et al.*, 2011; de Boer *et al.*, 2010; O'Leary *et al.*, 2009; Schuurmann *et al.*,

2007; Tam *et al.*, 2012). In this study, stored and concurrent sample testing resulted in *Campylobacter* culture sensitivity of 54–66%. Ascertainment of infection with *Salmonella* and *Shigella* in this study was also enhanced using these high-order multiplex molecular methods, mirroring closely other study findings (de Boer *et al.*, 2010; O'Leary *et al.*, 2009; Schuurmann *et al.*, 2007).

Very few laboratories routinely investigate all faeces for toxin-producing *Escherichia coli* other than for *Escherichia coli* O157, although the guidance in some countries recommends their detection in all clinical specimens (Gould *et al.*, 2009). Reference facilities in the UK request

Table 4. Pathogen detection from samples tested concurrently using conventional techniques and the Luminex GPP assay

Pathogen/target	No. detected	
	Conventional diagnostics*	Luminex GPP†
<i>Campylobacter</i>	22	34 (22)
<i>Clostridium difficile</i> toxin A/B	14	40 (14)
<i>Escherichia coli</i> O157	0	0
ETEC	–	1
STEC	–	8
<i>Salmonella</i>	1	1 (1)
<i>Shigella</i>	0	2
<i>Vibrio cholerae</i>	–	0
<i>Yersinia enterocolitica</i>	–	0
<i>Cryptosporidium</i>	0	0
<i>Giardia lamblia</i>	4	10‡ (4)
<i>Entamoeba histolytica</i>	0	0
Adenovirus 40/41	–	3
Norovirus	–	55
Rotavirus A	–	1
Total	41	155

*In total, 453/472 samples were processed for ova, cysts and parasites, and cultured for *Campylobacter*, *Escherichia coli* O157, *Salmonella* and *Shigella*, and 373 of these samples were tested for *Clostridium difficile* toxins.

†All 472 samples were tested using the Luminex GPP assay. Figures in parentheses correspond to the number of samples positive using conventional methods that were also detected using the Luminex and Savyon assays.

‡Nine of 10 samples confirmed using an alternative molecular assay.

submission of bloody faecal specimens for investigation, but results lack timeliness. The confirmed 2% detection rate in this study of STEC/EPEC using the Luminex GPP assay is comparable with rates published in other studies that also used non-culture techniques for their detection (Buvens *et al.*, 2012; Chui *et al.*, 2011; Couturier *et al.*, 2011; Gould *et al.*, 2009).

The clinical utility of PCR and other molecular techniques for the detection of *Clostridium difficile* infection remains a subject of intense debate. The >2-fold increase in *Clostridium difficile* detection with concurrent testing presented herein corroborates the evidence of independent publications that showed increased detection when compared with widely accepted algorithms (Chapin *et al.*, 2011; Culbreath *et al.*, 2012; Deshpande *et al.*, 2011; Eastwood *et al.*, 2009). Evidence is also emerging to suggest that molecular assays demonstrate good clinical accuracy, especially in hospitalized patients with suspected *Clostridium difficile* infection (de Jong *et al.*, 2012; Deshpande *et al.*, 2011; Kaltsas *et al.*, 2012). Furthermore, when compared with toxigenic culture (unpublished data), four of the five samples negative by toxigenic culture all had low median fluorescence intensity (MFI) values, suggesting a low burden of target organism as seen by authors investigating quantitative culture and PCR methods (Leslie *et al.*, 2012; Naaber *et al.*, 2011).

Accurate detection of protozoa in stools using conventional microscopy techniques is time-consuming and necessitates the use of highly trained personnel. The reported sensitivity of conventional detection of *Giardia* as compared with molecular methods is 51–78%, which is consistent with the approximate doubling of the detection rate for *Giardia* in both periods of testing using the Luminex GPP assay (de Boer *et al.*, 2010; Stark *et al.*, 2011; ten Hove *et al.*, 2009). Unconfirmed *Giardia* and *Entamoeba histolytica* positivity seen in the Luminex GPP assay has been described by other authors (Navidad *et al.*, 2013; Wessels *et al.* 2014). Due to the lack of *Cryptosporidium*-positive samples during both periods, a blinded panel of 15 *Cryptosporidium*-positive clinical samples provided by the UK *Cryptosporidium* Reference Unit demonstrated the capability of both assays to detect this parasite even in samples with low parasite load (unpublished data). The clinical significance of *D. fragilis* in faecal specimens is unresolved (Barratt *et al.*, 2011; Johnson *et al.*, 2004). Its presence as the sole potentially pathogenic micro-organism detected by either conventional or molecular means in 36/45 positive diarrhoeal samples by the Savyon assay may support its role in IID as maintained by several authors (Lagacé-Wiens *et al.*, 2006; Stark *et al.*, 2011).

It is acknowledged that the difference in storage time for the 1000 samples tested by both the Luminex and Savyon assays is a weakness of this study. This may have adversely affected the performance of the Savyon assay as samples that were detected by the Luminex assay and not the Savyon assay all had low MFI values. Another limitation of the study is the difference in sample input with the Luminex assay for the stored and concurrently tested

specimens. Nevertheless, regardless of sample input volume, the Luminex assay, which it should be noted tests for the largest number of organisms in comparison with the other assays in this study, detected more confirmed positives than conventional techniques in both sample sets.

The unconfirmed positivity seen in both assays may result from non-specific amplification due to the complex nature of high-order multiplex assays and of faecal specimens. Contamination can also not be ruled out as both assays require post-amplification handling. However, the micro-seal on the Savyon assay is not removed prior to introduction into the NanoChip platform, and the post-PCR handling for both Savyon and Luminex assays was performed in a dedicated post-amplification area with negative extraction and PCR controls consistently negative.

Use of both the Luminex GPP and Savyon GIP assays led to increased ascertainment of the aetiological agents of IID compared with conventional techniques with less hands-on time and reduced turn-around times for most pathogens. This is in keeping with the findings of other groups that have investigated the utility of syndromic tests (Claas *et al.*, 2013; Halligan *et al.*, 2013; Kahlau *et al.*, 2013). The increased detection rate for both assays resulted from increased sensitivity of detection of pathogens routinely sought, but also from testing for additional pathogens compared with conventional methods, as demonstrated by other authors (Claas *et al.*, 2013; Kahlau *et al.*, 2013; Mengelle *et al.*, 2013).

The economic value of the additional detections provided by such molecular syndromic strategies is not clear although study data are emerging that addresses their potential economic viability (Halligan *et al.*, 2013). Consumable costs are certainly greater for molecular detection compared with culture, microscopy and ELISA; however, when multiple tests are applied to samples, overall costs may be similar if staff time and grade are taken into account. In addition, where laboratories already perform commercial *Clostridium difficile* and/or norovirus NAATs, the inclusion of one or both of these targets in large multiplex panels may lead to further efficiencies. However, overall laboratory expenditure is likely to increase.

In this study setting only diarrhoeal stool samples were tested routinely, mitigating concerns about detection of pathogens in asymptomatic individuals. Nevertheless, the clinical relevance of NAAT positive-culture/toxin ELISA-negative detections is a moot point. The ability to 'switch off' reporting of specific pathogens or groups of pathogens with the Luminex GPP assay, such as for *Clostridium difficile* in infants (<2 years) and community patients, could be seen as advantageous. It should be remembered, however, that although clinicians and scientists have become accustomed to interpreting previously available diagnostic results in defined patient groups, the methods and strategies by which they are obtained are not perfect. Indeed, the perceived strengths and weaknesses of both current and emerging methodologies used in isolation may lead to a distorted view of the clinical spectrum of disease,

and the use of both (e.g. employing culture and ELISA to detect viable organism/toxin subsequent to NAAT positivity) for an interim period, or indefinitely, might be clinically judicious. The possibility for NAATs to inform earlier interventions, which could improve patient outcome on an individual and/or population level, must also not be disregarded at this point in spite of the inevitable impact of increased detections on hospital and community healthcare professionals.

In summary, it has been shown that both the Luminex GPP and Savyon GIP assays were able to accurately detect 85/90 pathogens/toxins detected using conventional methods from 1000 consecutive stored samples tested in a routine clinical enteric laboratory. In addition, both assays were shown to detect a large number of positive samples that were negative by conventional means due to the insensitive techniques employed and/or deficiencies in testing algorithms. The Luminex GPP assay identified all intestinal pathogens encountered using conventional techniques in 472 liquid stool samples over a 2 month period when samples were tested concurrently with increased sample input volume (200 versus 100 µl). During this period the Luminex GPP assay also further identified additional pathogens as seen in the stored sample testing. Molecular assays could contribute to increased workflow efficiency in laboratories and clinics, despite the fact that confirmatory testing may be warranted for some targets. It is imperative that the clinical relevance and economic impact of employing such assays is investigated further.

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