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Evaluation of a multiplex real-time PCR kit Amplidiag® Bacterial GE in the detection of bacterial pathogens from stool samples

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ABSTRACT

This study evaluated the performance of a new commercially available multiplex real-time PCR kit Amplidiag® Bacterial GE in the systematic screening of bacterial pathogens causing gastroenteritis. Stool samples from 1168 patients were analyzed with Amplidiag® Bacterial GE, stool culture, and molecular reference tests, and the sensitivity and specificity of Amplidiag® Bacterial GE were determined by comparing the results to the reference tests. The evaluation showed good performance for Amplidiag® Bacterial GE: sensitivity and specificity of the test was 100/99.7% for *Salmonella*, 100/99.8% for *Yersinia*, 98.8/99.2% for *Campylobacter*, 92.9/100% for *Shigella*/EIEC, 100/99.9% for EHEC, 92.9/99.8% for ETEC, 98.9/99.2% for EPEC, and 100/99.8% EAEC, respectively. When compared with stool culture, Amplidiag® Bacterial GE was found to be more sensitive. This study suggests that Amplidiag® Bacterial GE is suitable for screening bacterial pathogens from stool samples. However, this study only demonstrates the performance of Amplidiag® Bacterial GE in low endemic settings, as the number of positive findings in this study was relatively low.

1. Introduction

There are almost 1.7 billion cases of diarrhea in the world every year (World Health Organization (WHO), 2013). Diarrhea is the second leading cause of child morbidity and mortality in the developing countries, and acute diarrhea is one of the most common illnesses for travelers (World Health Organization (WHO), 2013; Kelly, 2011). Fast and reliable diagnosis of gastroenteritis is important for ensuring proper management of the diarrheal patients. If not treated, some bacterial pathogens are able to develop persistent gastrointestinal symptoms (Arenas-Hernández et al., 2012; Connor, 2013; World Health Organization, 2005). In addition, *Campylobacter*, *Salmonella* and *Shigella* are strongly linked to the development of reactive arthritis, yet the effect of proper treatment of gastroenteritis on the incidence of arthritis is still unclear (Ajene et al., 2013). Then again, in many instances antimicrobials are unnecessarily prescribed for self-limited diarrhea, leading to higher risk of colonization with antimicrobial resistant intestinal bacteria (World Health Organization, 2005; Kantele et al., 2015). In addition, antibiotics can trigger haemolytic uremic syndrome, a life-threatening condition linked to Shiga toxin producing *Escherichia coli* (Tarr et al., 2005; Wong et al., 2012).

When suspecting bacterial gastroenteritis, bacterial culture is the standard procedure for diagnostics (Bennett and Tarr, 2009; Farthing and Kelly, 2007). Conventional bacterial culture is relatively reliable method for systematic screening of bacterial pathogens from stool samples, but it usually takes several working days to get the final results. In addition, conventional methods are able to detect only a few causative agents of diarrhea (Fhogartaigh and Dance, 2013). In recent years, molecular methods for clinical diagnostics have quickly emerged and improved our understanding on the epidemiology of bacterial gastroenteritis (Fhogartaigh and Dance, 2013; Zhang et al., 2015). For gastrointestinal pathogen detection, multiplex PCR based methods are considered most promising. Currently available multiplex PCR assays differ notably in their detection panels, assay turnaround time and protocol complexity (Zhang et al., 2015). In comparison to conventional diagnostic methods, the majority of the molecular methods have lower hands-on time, simpler result interpretation and higher sensitivity (Zhang et al., 2015; de Boer et al., 2010; Biswas et al., 2014; Anderson et al., 2014).

Amplidiag® Bacterial GE is a novel multiplex real-time PCR kit designed to detect the most common and important bacteria causing bacterial gastroenteritis. The test kit has been developed based on experience from an in-house quantitative PCR test that has been originally designed for rapid diagnostics of acute travelers' diarrhea, but also possesses suitability for systematic screening of bacterial pathogens

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from stool samples of patients suffering from different gastrointestinal symptoms (Antikainen et al., 2013). The PCR assay panel includes eight pathogen targets divided in three multiplex reactions (Table 1). With Amplidiag® Bacterial GE, pathogens can be detected directly from DNA isolated from stool samples without the need for stool culture. The results are gained within a few hours after the sample arrival in laboratory.

This study analyzed the suitability of Amplidiag® Bacterial GE in systematic screening of bacterial pathogens from clinical specimens of patients with distinct gastrointestinal symptoms. The study was conducted in two clinical microbiology laboratories, one situated in Turku, Finland, and the other in Stockholm, Sweden. Stool samples from 1168 patients were analyzed with Amplidiag® Bacterial GE, stool culture and molecular biology reference tests, and the sensitivity and specificity of Amplidiag® Bacterial GE were determined by comparing the results to the biochemical and molecular methods.

2. Materials and methods

2.1. Sample collection

Stool samples from 1168 individual patients were included in this study. Samples were originally sent to a clinical microbiology laboratory for stool culture between April 7th 2014 and June 17th 2014. Four hundred and seventy-one samples were collected in Turku while 697 samples were collected in Stockholm. Ethical approval for using the patient samples was not required, as the study was considered as a basic laboratory method development for the same, original diagnostic purpose. No additional sample container or additional information was collected, and no other inconvenience for patients was generated. Only the stool culture results were reported to customers.

In Turku, clinical specimens were collected in regular stool jars without preservatives, while in Stockholm, samples were collected in eSwab collection tubes (Copan, Italy). Specimens arriving in the laboratory were cultured and then stored at +4 °C. In Turku, samples older than three days were excluded from the study to eliminate bias of false negative culture results due to logistical issues. In addition, samples arriving in the laboratory on Friday were excluded from the study to avoid bias caused by a long DNA extraction delay.

The age of the study subjects ranged from 0 to 100 (mean 46 years), and the sex distribution was 44% males and 56% females. Clinical history for the majority of the samples was not available, but as the samples were originally sent for stool culture, it can be presumed that the patients either suffered from gastrointestinal symptoms or had recently received treatment for a gastrointestinal condition.

Table 1
Amplidiag® Bacterial GE assay panel.

	Origin	Gene	Channel
Multiplex 1	EHEC	*stx1*, *stx2*	Green
	EHEC/EPEC	*eae*	Yellow
	Salmonella spp.	*invA*	Orange
	Amplification control		Red
Multiplex 2	ETEC	*est*, *elt*	Green
	Yersinia enterocolitica/pseudotuberculosis/pestis	*rumB*, *virF*	Yellow
	Campylobacter jejuni/coli	*rimM*, *gyrB*	Orange
	Amplification control		Red
Multiplex 3	*Shigella*/EIEC	*ipaH*, *invE*	Green
	EAEC	*aggR*	Yellow
	Amplification control		Red

Abbreviations: EAEC, enteroaggregative *E. coli*; EHEC, enterohaemorrhagic *E. coli*; EIEC, enteroinvasive *E. coli*; EPEC, enteropathogenic *E. coli*; ETEC, enterotoxigenic *E. coli*.

2.2. Evaluation workflow

All patient samples (n = 1168) were analyzed with stool culture, Amplidiag® Bacterial GE (Mobidiag Ltd., Finland), and molecular reference tests: RIDA®GENE Bacterial Stool Panel (R-Biopharm AG, Germany), genesig® *Campylobacter jejuni* and *Campylobacter coli* kits (Primerdesign Ltd., UK), and an in-house multiplex PCR for the diarrheagenic *E. coli* species (Antikainen et al., 2009). In the result analysis, stool culture was considered as the primary reference method for the identification of *Salmonella*, *Campylobacter*, *Shigella* and *Yersinia*. However, if discrepancies between stool culture and Amplidiag® Bacterial GE results occurred, molecular reference tests were considered as the gold standard. In these cases, RIDA®GENE Bacterial Stool Panel (R-Biopharm AG, Germany) was the reference test for *Salmonella* and *Yersinia* identification, while genesig® *Campylobacter jejuni* and *Campylobacter coli* kits (Primerdesign Ltd., UK) were the reference tests for *Campylobacter* identification. As no culture methods for *E. coli* identification were included in this study, the in-house multiplex PCR for diarrheagenic *E. coli* species (Antikainen et al., 2009) was considered as the gold standard for EAEC, EHEC, EIEC, EPEC, and ETEC detection.

2.3. Nucleic acid extraction

Samples included in the Amplidiag® Bacterial GE method evaluation were recorded with Amplidiag ID numbers and the nucleic acid (NA) extraction was performed on the following morning. An extraction control, representing cultured *Salmonella enterica* spp. *enterica* serovar Newport (ATCC 6962) cells diluted in PBS, was included in each extraction series to control the performance of the NA extraction instrument. In Turku, bacterial NA from stool samples was extracted with MagNA Pure 96 DNA and Viral NA Large Volume Kit (Roche Diagnostics GmbH, Germany) following the manufacturer's protocol. Prior to extraction, 150–200 mg of sample material was suspended in 600 μl of 1 × PBS, heated at 100 °C for 10 min, and centrifuged 15 s at 500 ×*g*. 300 μl of the supernatant was mixed with 250 μl of MagNA Pure Bacteria Lysis Buffer (Roche Diagnostics GmbH, Germany) and 500 μl of the solution was used for the extraction. The final elution volume after extraction was 100 μl. In Stockholm, bacterial NA was extracted with semiautomatic Bullet Stool Kit (DiaSorin, Ireland) following the manufacturer's instructions. Sample material used for the extraction was 400 μl, and the final elution volume was 100 μl. The NA eluates were divided into several aliquots, of which one was analyzed with Amplidiag® Bacterial GE without delay, while the others were stored at −70 °C until analysis with molecular reference tests.

2.4. Multiplex real-time PCR

Amplidiag® Bacterial GE PCR was performed according to the manufacturer's instructions. The assay panel included eight diarrhea-causing bacterial pathogens divided into three multiplex reactions (Table 1). Master Mix and Assay Mixes 1–3 were ready-to-use. The amount of template DNA in one reaction was 5 μl, and the total reaction volume was 25 μl. Each PCR series included an extraction control, a positive control mix including templates for all tested targets, and a negative control. In addition, each reaction included an internal amplification control for the detection of the presence of PCR inhibitors.

In Turku, amplification reactions were performed with CFX96 (Bio-Rad). In Stockholm, PCR was done with ABI 7500 FAST qPCR instrument (Applied Biosystems). Twenty-nine samples could be analyzed in one PCR run when using 96-well-plates. The raw data was exported to and analyzed by a fully automated analysis program Amplidiag® Analyzer (Mobidiag Ltd., Finland). The software normalized minor variations between reagent batches and different qPCR instruments for data interpretation. The program reported the results in written format, including potential warnings from extraction controls, positive and negative controls, and internal amplification controls. The Amplidiag®

Bacterial GE analysis workflow, including NA extraction, PCR, and result interpretation, took approximately 3 h in total, when up to 29 stool samples were simultaneously analyzed.

3. Results

When the combined results of stool culture and molecular reference tests were considered as a gold standard, the sensitivity and specificity of Amplidiag® Bacterial GE for clinical stool specimens was 100/99.7% for *Salmonella*, 100/99.8% for *Yersinia*, 98.8/99.2% for *Campylobacter*, 92.9/100% for *Shigella*/EIEC, 100/99.9% for EHEC, 92.9/99.8% for ETEC, 98.9/99.2% for EPEC, and 100/99.8% EAEC, respectively (Table 2). All Amplidiag® Bacterial GE results that went along with the gold standard results were defined as true positive or true negative. Amplidiag® Bacterial GE results that could not be confirmed by the reference methods were determined as false positive or false negative.

Of the 1168 clinical patient samples, 914 (78.3%) were negative for all studied pathogens with both Amplidiag® Bacterial GE and the gold standard. One hundred seventy-three samples (14.8%) were positive for one pathogen, 46 (3.9%) for two pathogens and 13 (1.1%) for three or more pathogens simultaneously. EPEC and *Campylobacter* were the most common findings; 8.0% of all samples were EPEC positive while 7.3% were *Campylobacter* positive.

Two hundred thirty-seven (20.3%) of the 1168 clinical specimens were positive for one or more studied bacterial pathogens with the reference methods. With Amplidiag® Bacterial GE, the number of positive samples was 249 (21.3%). When considering the reference methods being the gold standard, 17 samples would have been diagnosed incorrectly positive with Amplidiag® Bacterial GE: 7 EPEC, 4 *Campylobacter*, 2 *Salmonella*, 2 *Yersinia*, EHEC, and EAEC. In addition, nine samples positive for one pathogen with both Amplidiag® Bacterial GE and the reference methods, had an additional positive call only with Amplidiag® Bacterial GE (5 *Campylobacter*, 2 EPEC, *Salmonella*, ETEC), one sample positive for two pathogens had an additional positive call for *Salmonella* with Amplidiag® Bacterial GE, and one sample positive for two pathogens had two additional positive calls (ETEC, EAEC) with Amplidiag® Bacterial GE. In total, the number of these additional positive calls with Amplidiag® Bacterial GE was 29. When considering the molecular reference methods being the gold standard, five samples were incorrectly negative with Amplidiag® Bacterial GE: 2 ETEC, EPEC, *Campylobacter*, and *Shigella*/EIEC. However, when compared with stool culture, no false negative results were detected.

With stool culture, 72 of the 1168 studied clinical specimens were reported positive: 11 samples were positive for *Salmonella*, 57 for *Campylobacter*, two for *Yersinia* and three for *Shigella* (Fig. 1). The total number of findings was 73, as one sample was positive for both *Salmonella* and *Campylobacter*. When considering the Amplidiag® Bacterial GE results that went along with the molecular biology reference tests, the number of positive specimens was 232 and the total number of findings was 308 (Fig. 2). Of these findings, 16 were *Salmonella*, 84 *Campylobacter*, 2 *Yersinia*, and 13 *Shigella*/EIEC, the total number of findings for pathogens traditionally analyzed only with stool culture being 115.

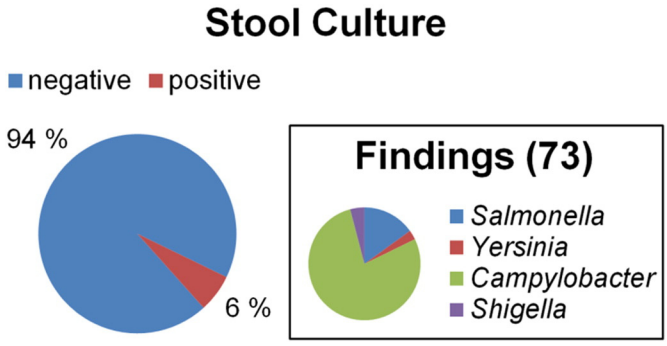

Fig. 1. The bacterial pathogen findings with conventional stool culture. The number of samples reported positive with stool culture was 72 (6%), and the total number of finding was 73: 11 *Salmonella*, 2 *Yersinia*, 57 *Campylobacter*, and 3 *Shigella*.

4. Discussion

Based on this study, Amplidiag® Bacterial GE multiplex real-time PCR kit performs well in the qualitative detection and identification of the most prominent bacterial pathogens associated with gastroenteritis. The kit is easy-to-use, and hands-on time needed is low in comparison to traditional stool culture. The key finding of this study is that Amplidiag® Bacterial GE does not compromise sensitivity when compared to traditional stool culture. In fact, when considering the number of *Salmonella* and *Campylobacter* findings during the evaluation, Amplidiag® Bacterial GE clearly rises above stool culture in capability to identify these pathogens from the patient samples; a total of 32 samples found positive for *Salmonella* or *Campylobacter* with both Amplidiag® Bacterial GE and the molecular reference methods remained negative in conventional stool culture. When compared to the consolidated gold standard results, Amplidiag® Bacterial GE presented altogether 5 false negative and 29 false positive calls for the studied pathogens. However, apart from a single false identification of *C. upsaliensis* as *C. coli*, the rest of the supposed false positive results might actually originate from an inability to confirm the low abundant findings with the molecular reference methods. This study thus shows good performance for Amplidiag® Bacterial GE. However, this study only demonstrates the performance of the method in low endemic settings, as the overall number of the positive findings was relatively low. This is probably due to the occasion of the sample collection: the study was performed in late spring, while in general the bacterial gastroenteritis is most prevalent in the warm season, which in Finland and Sweden is basically limited to summer months July and August.

Table 2
Final results of the Amplidiag® Bacterial GE performance evaluation.

Target	Gold Standard[a]		Amplidiag® Bacterial GE					
	Positive	Negative	True positive[b]	True negative[b]	False positive[c]	False negative[c]	Sensitivity	Specificity
EHEC	7	1161	7	1160	1	0	100.0%	99.9%
EPEC	94	1074	94	1065	9	1	98.9%	99.2%
Salmonella	16	1152	16	1148	4	0	100.0%	99.7%
ETEC	28	1140	26	1138	2	2	92.9%	99.8%
Yersinia	2	1166	2	1164	2	0	100.0%	99.8%
Campylobacter	85	1083	84	1074	9	1	98.8%	99.2%
Shigella/EIEC	14	1154	13	1154	0	1	92.9%	100.0%
EAEC	66	1102	66	1100	2	0	100.0%	99.8%

Abbreviations: EAEC, enteroaggregative *E. coli*; EHEC, enterohaemorrhagic *E. coli*; EIEC, enteroinvasive *E. coli*; EPEC, enteropathogenic *E. coli*; ETEC, enterotoxigenic *E. coli*.
[a] Combination of the results of stool culture and molecular reference methods.
[b] Amplidiag® Bacterial GE consistent with the gold standard results.
[c] Amplidiag® Bacterial GE not consistent with the gold standard results.

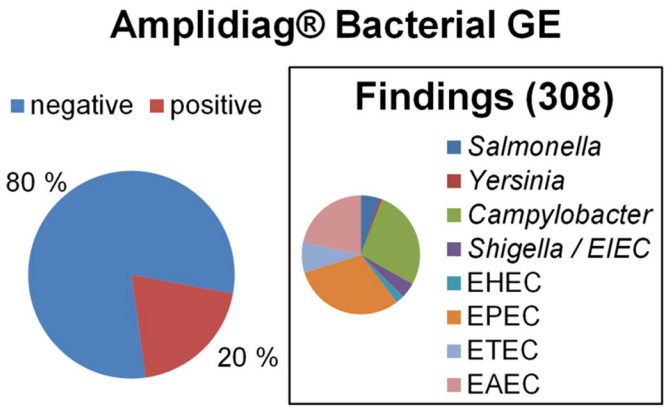

Fig. 2. The bacterial pathogen findings with Amplidiag® Bacterial GE. Only the results confirmed by the molecular reference tests are presented. The number of positive samples was 232 (20%), and the total number of findings was 308: 16 *Salmonella*, 2 *Yersinia*, 84 *Campylobacter*, 13 *Shigella*/EIEC, 7 EHEC, 94 EPEC, 26 ETEC, and 66 EAEC.

When compared with other commercial multiplex real-time PCR kits (Zhang et al., 2015), the main advantages of Amplidiag® Bacterial GE include fast turnaround time with high throughput, simple result interpretation, and detection of all diarrheagenic pathotypes of *E. coli* among other important bacterial pathogens. Even though certain commercial multiplex PCR tests (Spina et al., 2015; Harrington et al., 2015) are undoubtedly more user-friendly than Amplidiag® Bacterial GE and other non-integrated qPCR methods, the random access methods often either possess a limited test panel (Harrington et al., 2015), or are more suitable for stand-by 24/7 diagnostics rather than screening of large sample cohorts due to low throughput (Spina et al., 2015). The general downside of all currently available molecular tests is the lack of antibiotic resistance information and exact species level identification. To gain these, stool culture and traditional biochemical methods are still required. However, the resistance testing is not necessary for most gastroenteritis pathogens. Furthermore, stool culture could be performed after gaining the PCR results, for positive samples only.

Currently many clinical microbiology laboratories routinely diagnose EHEC serotype O157:H7 by bacterial culture, but few laboratories utilize antigen detection or molecular methods to additionally detect non-O157:H7 EHEC (Marcon, 2011). In addition, other pathogenic *E. coli* variants are often completely overleaped, even though the prevalence of various pathotypes is notable in diarrheal patients (Marcon, 2011). PCR tests for the identification of diarrheagenic *E. coli* subtypes have been developed, but these assay panels are often limited to *E. coli* alone (Antikainen et al., 2009; Persson et al., 2007). In fact, the majority of otherwise comprehensive commercial multiplex real-time PCR panels exclude at least some of the diarrheagenic *E. coli* variants (Zhang et al., 2015). Thus, the ability to detect all diarrheagenic *E. coli* pathotypes with Amplidiag® Bacterial GE is a huge advance.

Detection of *Campylobacter* in multiplex PCR panels is often somewhat overwhelming; some panels detect *Campylobacter* spp., meaning that the positive results can represent non-pathogenic *Campylobacter* species as well as pathogenic (Zhang et al., 2015). Amplidiag® Bacterial GE detects the most prevalent pathogenic species *C. jejuni* and *C. coli*, excluding all non-pathogenic species. This makes the result interpretation easy, but the downside is that rare pathogenic species like *C. lari* and *C. fetus* are also ignored. For *Yersinia enterocolitica* detection, Amplidiag® Bacterial GE exploits primers specific to a plasmid-borne *virF* gene. As *virF* is a virulence gene, Amplidiag® Bacterial GE recognizes only the virulent strains of the bacterium. This feature is valuable in diagnostics, as non-pathogenic *Y. enterocolitica* stains are not detected. Yet, for this same reason, the performance of Amplidiag® Bacterial GE in *Yersinia* diagnostics cannot be tested with commercial quality control species, as the virulence plasmid is often missing from the control strains either purposely or due to extensive *in vitro* cultures (Zheng et al., 2008). One possible weakness of Amplidiag® Bacterial GE is the lack of *Aeromonas* in the assay panel. The role of *Aeromonas* as a potential enteric pathogen is still debated though (Janda and Abbott, 2010), therefore justifying the exclusion of the bacterial genus from the assay.

Bacterial culture from stool is unquestionably less expensive than molecular screening, when looking only at the analytical cost of an individual sample. Nevertheless, the hands-on time saved in the laboratory and the decrease in the answering delay could lower the total cost burden of the health care system. Analyses between conventional stool culture and commercial multiplex PCR methods have already given promising results (Halligan et al., 2014; Mortensen et al., 2015), and for example in *Clostridium difficile* testing, fast molecular biology methods have already been found more cost-effective than traditional methods in hospital-wide scale (Schroeder et al., 2014). However, more comprehensive economic studies should be performed to reveal the real cost savings and overall impact of using molecular biology methods in the clinical process of gastroenteritis diagnosis.

5. Conclusions

This study demonstrates that Amplidiag® Bacterial GE kit is a valuable tool in the screening of bacterial pathogens from diarrheal stool samples, at least in low endemic settings. When compared to traditional stool culture, Amplidiag® Bacterial GE is evidently faster and needs less hands-on time, without compromising sensitivity. To take the full advance out of fast and accurate molecular biology methods, clinical laboratories should be progressive enough to change their routine diagnostic traditions.

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