Evaluation of the Seegene PCR for detection of enteric pathogens

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Seegene Allplex™ gastrointestinal assay was easy to perform as all panels were run on the same platform and only needed one extraction to detect all included microorganisms. With this selected material of fecal samples known to be positive for enteric pathogenic bacteria, enteric pathogenic *E. coli* and enteric pathogenic protozoans the Seegene assay detected most microorganisms and had a high sensitivity even though some *Entamoeba histolytica* were not detected. With the selected fecal samples known to be negative for protozoans the Seegene assay found few false positives and had a good specificity. With the selected fecal samples known to be negative for enteric pathogenic bacteria and enteric pathogenic *Escherichia coli* a substantial number of false positive samples were found, which resulted in a low specificity. Some of these results could be explained by a suboptimal culture technique. Thus, several of these false positive samples had high C(t) values which may be caused by a low number of bacteria or an unspecific reaction. Further studies on consecutive patient material are needed to get more reliable data on the true false positive rate.

Keywords: faecal culture; faecal RT-PCR; protozoan; pathogenic *E. coli*; enteric pathogenic bacteria.

1. Introduction

The etiological agents for infectious diarrhoea are diverse and usually impossible to distinguish by the clinical appearance. Furthermore, the identification of the pathogenic agent may be difficult because of the presence of large amounts of non-pathogenic microorganisms present in the stool[1, 2]. It is therefore of great importance to use a quick and accurate diagnostic method to identify the potential pathogenic microorganisms. The golden standards for identification of pathogenic microorganisms in stool samples have previously been culture of bacteria and microscopy of parasites. However, these techniques may differ considerably from laboratory to laboratory. Culture of stool samples on several different media is time consuming and the result may be delayed for several days[3]. One specimen is usually sufficient for the detection of most bacterial pathogens. However, submitting a second specimen has been shown to increase the overall sensitivity of the test by 20%[4]. Treatment decisions are usually based in the clinical severity of illness prior to receipt of culture confirmation of microbial agent[5].

Diagnostic methods that might reduce the time could be molecular biological methods for initial screening of the clinical samples. It has been shown in multiple studies that the value of adding molecular multiplex detection of enteric pathogens is superior to conventional methods[6-8]. For detection of gastrointestinal pathogens, multiplex polymerase chain reaction (PCR) based methods are considered most promising[7]. Both in-house and commercial multiplex real-time PCR (RT-PCR) are described in the literature detecting many different enteric pathogenic microorganisms[6-10] The published detection levels for culture of foodborne pathogenic bacteria in faeces are $>10^6$ CFU/g and samples that were positive by the RT-PCR assay and negative by culture showed high C(t) values[6]. It was concluded that these samples contained few target bacteria $< 10^3$ CFU/g[6]. Several companies offer RT-PCR assays for enteric pathogenic microorganisms and there is a rapid progress in the field. Several limitations of molecular assays should be kept in mind. Since these tests are based on the detection of specific genetic targets, they are incapable of detecting pathogens for which a target is lacking. Another criticism of molecular assays is that they leave a laboratory without an isolate for susceptibility testing and typing, which is of epidemiological importance[11]. Other species that may cause severe diarrhoea such as non-thermophilic *Campylobacter*, enteropathogenic *Clostridium perfringens*, *Laribacter hongkongensis*, enteropathogenic *Helicobacter* species and *Archobacter* species are not included in any commercial assay.

Some of the commercial assays, such as FilmArray® (22 different pathogens) (Biomerieux), only take one sample in a run and are not useful for large scale routine diagnosis. These methods are very rapid and useful for single critically ill patients. Other assays, such as BD max™ Gastrokit (4 species) and Extended Gastrokit (4 species) and Amplidiag® Bacterial GA (10 species divided on 3 panels), can take several samples in a run, but include a limited number of enteric pathogenic microorganisms.

Seegene Allplex™ gastrointestinal assay cover a broad spectrum of enteric pathogens divided in four panels: Panel 1: detects six different enteric pathogenic viruses, Panel 2 (Bacteria I): detects the most common pathogenic bacteria, panel 3 (Bacteria II): detect toxin producing *Escherichia coli* and *Clostridium difficile* 027, and panel 4: detects six different species of protozoans (Table 1). All four panels are amplified using the same PCR thermal programme, which makes it possible to run the analyses simultaneously for all panels.

The purpose of this study was to compare Seegene PCR Allplex™ Gastrointestinal panel assay (bacteria I, bacteria II and parasite panels) with classical culture and microscopy of known positive and negative faecal samples.

Table 1 Seegene Allplex™ gastrointestinal assay panel

Panel 1 (Virus)	Panel 2 (Bacteria I)	Panel 3 (Bacteria (II)	Panel 4
Norovirus GI	*Campylobacter* spp.	*Clostridium difficile* hypervirulent	*Giardia lamblia*
Norovirus GII	*Clostridium difficile* toxin B	*E. coli* O157	*Entamoeba histolytica*
Rotavirus	*Salmonella* spp.	EHEC* (*stx1/2*)	*Cryptosporidium* spp.
Adenovirus	EIEC*/*Shigella* spp.	EPEC* (*eaeA*)	*Blastocystis hominis*
Astrovirus	*Vibrio* spp.	ETEC* (*lt/st*)	*Dientamoeba fragilis*
Sapovirus	*Yersinia enterocolitica*	EAEC* (*aggR*)	*Cyclospora cayetanensis*
	Aeromonas spp.		

*EIEC: Enteroinvasive *E. coli*, EHEC: Enterohemorrhagic *E. coli*, EPEC: Enteropathogenic *E. coli*, ETEC: Enterotoxigenic *E. coli*, EAEC: Enteroaggregative *E. coli*

2. Materials and Methods

2.1 Samples

The faecal samples were cultured for enteric pathogenic bacteria or examined for protozoans by microscopy of sediments obtained by the Ridley concentration method. Positive and negative samples were collected over a six month period since the number of positive samples was rather low. Therefore, samples were stored at -80°C until the DNA extraction for RT-PCR. The positive protozoan samples were collected from 2009-2014 since positive samples are quite rare in our department.

The faecal samples were cultured at 37°C on lactose agar plates, 5% horse blood agar plates and SSI enteric medium plates under aerobic conditions, on cycloserine-cefoxitin-fructose agar (CCFA) plates under anaerobic conditions and on *Campylobacter* charcoal differential agar (CCDA) plates under microaerobic conditions (5% O_2). *Escherichia coli* from patients suspected to have pathogenic *E. coli* were serotyped and sent to the National Reference Laboratory at Statens Serum Institut.

The positive samples were divided into three groups: i) 19 faecal samples culture positive for enteric pathogenic bacteria, ii) 44 strains of toxin-producing *E. coli* were added to a faecal sample, which was negative for enteric pathogenic bacteria and protozoans (both by normal routine investigation and Seegene Allplex™ gastrointestinal assay).

The *E. coli* strains were selected to have different genes and gene combinations (Table 2). iii) 47 faecal samples, which were found positive for protozoans by microscopy. *Entamoeba histolytica* and *E. dispa*r were discriminated by an in-house RT-PCR on BD-MAx™ using DNA extraction ExK DNA-4 kit.

The same grouping was used for the negative faecal samples: i) 72 faecal samples culture negative for enteric pathogenic bacteria, ii) 90 faecal samples culture negative for toxin-producing *E. coli* and iii) 46 faecal samples negative for protozoans by microscopy.

2.2 DNA extraction and PCR

The frozen faecal samples were placed on ice to ensure that they did not thaw before DNA extraction. The DNA extraction was performed using the QIAamp fast DNA stool mini kit (QIAgen, Germany) according to the manufactures guidelines. The Seegene Allplex™ gastrointestinal assay contains an internal control, which must be added to the DNA extraction procedure to ensure good performance. The internal control was added to the samples before proteinase K treatment in the DNA extraction protocol.

The three tested assays are all identical in the PCR master mix composition and the PCR thermal programme, which makes it possible to run all three assays simultaneously. The PCR master mix composition for each sample was: 5 µL 5X GI-XX MON (MuDT oligo Mix), 5 µL 5X anyplex PCR Master Mix (DNA polymerase, Uracil-DNA glycosylase (UDG) and dNTPs) and 10 µL RNase-free water (Seegene, Inc.). 5 µL of each sample was added to the master mix. The real-time PCR was performed using CFX96™ (Bio-Rad Laboratories, Inc.) where the programme Bio-Rad CFX Manager (Bio-Rad) records the raw data. Afterwards the data was exported and opened for analysis in the Seegene Viewer program (Seegene, Inc.). The Seegene Viewer analyses the data automatically and indicate by a red dot if the sample is positive.

Table 2 The gene combinations of the different pathogenic *E. coli* used in the study.

OTYPE	***vtx1***	***vtx2***	***vtx2f***	***eae***	***estA*$_{H}$**	***estA*$_{P}$**	***elt***	***ipaH***	***aggR***
O172	-	-		-	-	-	-	+	
O128ab	-	+	-	-				-	
O124	-	-		-	-	-	-	+	
O 39	-	-		-	+	-	+	-	
O8	-	-		-	-	+	-	-	
O157	+	+		+					-
O127	-	-		+					
O26	+	-		+	-	-	-	-	
O3	-	-		-	-	-	-	-	+
O44	-	-		-	-	-	-	-	+
O78					+	+	+		
O104	-	-	-	-				-	+
O174	-	+		-			-	-	
O121	-	-		+	-	-	-	-	
O6	-	+		-					
O118	-	+		-					
O165	-	+	-	+				-	
O101	-	+	-	-				-	
O145	-	-		+	-	-	-	-	
O103	-	-		+	-	-	-	-	
O92	-	-		-	-	-	-	-	+
O153	-	-	-	-	-	-	-	-	+
O174	+	+	-	-				-	
O121	-	-		-	-	-	-	+	
O128ac	-	-	+	+					
O111	+	-		+					
O8	+	-	-	-	-	-	-	-	
O2	-	+	-	-	-	+	-	-	
O177	-	+	-	+				-	
O 40	-	+	-	-				-	
O157	-	-		+					-
O26	-	-		+			-		-
O157	+	+		+					-
O41	-	+		+			-		-
O26	-	+	-	+			-	-	-
O111	+	-	-	+			-	-	-
O6	-	-	-	-	-	-	+	-	-
O166	-	+	-	-	-	-	+	-	-
O63	-	+	+	+			-	-	-
O104	+	-	-	-	-	-	-	-	-
O121	-	+	-	+			-	-	-
O174	-	+	-	-			-	-	-
O157	-	+	-	+			-	-	-

3. Results

3.1 Enteric pathogenic bacteria

The Seegene bacteria I detect *Shigella*/EIEC, *Campylobacter jejuni/coli*, *Salmonella* spp., *Yersinia enterocolitica*, *Vibrio cholera/vulnificus*, *Aeromonas* spp., *C. difficile* and was tested on 19 culture positive samples. Eighteen of 19 culture positive samples were found positive with using this panel. One sample with *Salmonella agona* gave a negative result. This pathogen is not included in the panel. The panel found additionally 11 microorganisms (seven *Campylobacter* spp., three *Shigella*/EIEC, one *Aeromonas* spp.) in seven culture positive samples (Table 3).

The Seegene bacteria I panel found 21 of 72 faecal samples without cultured enteric pathogenic bacteria positive for enteric pathogenic bacteria: eight *Shigella*/EIEC, seven *Campylobacter* spp., three *Yersinia enterocolitica*, two *Clostridium difficile* and one *Salmonella* spp. (Table 3).

Table 3. Performance of Seegene RT-PCR bacteria panel I compared with culture for enteric pathogen bactreria.

	Positive samples (19)		Negative samples (72)	
Bacteria	Culture	Bacteria I panel	Culture	Bacteria I panel
Shigella/EIEC	3	6	0	8
Campylobacter spp.	9	16	0	7
Salmonella spp.	3	2	0	1
Yersinia enterocolitica	3	3	0	3
Vibrio spp.	0	0	0	0
Aeromonas spp.	1	2	0	0
Clostridium difficile	2	2	0	2

3.2 Enteric pathogenic *E. coli*

The Seegene bacteria II panel detected the following pathogenic *E. coli: stx1/2* (STEC), *eae*A (EPEC), *aggR* (EAEC), O157 (STEC). Since the routine culture found very few enteric pathogenic *E. coli,* pure culture of enteric pathogenic *E. coli was* mixed with a known negative faecal sample. The Seegene bacteria II panel found pathogenic *E. coli* in 43 of 44 faecal samples with known toxin producing *E. coli* culture. Seegene bacteria II found additionally 42 *E. coli* genes in the positive samples (Table 4). The one sample positive for *stx1/2*, which the Seegene bacteria II did not find had the gene combination *vtx2* positive, *vtx2f* positive and *eaeA* positive. Furthermore, the serotype was O63.

In 90 faecal samples culture negative for pathogenic *E. coli*, 40 were found positive by the Seegene bacteria II assay, Table 4.

Table 4. Performance of Seegene RT-PCR bacteria panel II compared with culture for enteric pathogenic *E. coli*.

E. coli gene	Pathogenic *E. coli* from SSI (44)		Culture negative samples (90)
	Seegene bacteria II positive/actual number of positives	SSI negative/Seegene negative	Seegene bacteria II positive/actual number of negatives
stx1/2 (STEC)	24/25 (96%)	4/19 (21%)	10/90 (11%)
lt/st	4/4 (100%)	30/40 (75%)	8/90 (9%)
O157 (STEC)	4/4 (100%)	36/40 (90%)	1/90 (1%)
eaeA (EPEC)	10/10 (100%)	25/34 (74%)	12/90 (13%)
aggR (EAEC)	6/6 (100%)	34/38 (89%)	9/90 (10%)

3.3 Parasites

The Seegene parasite panel detected *E. histolytica*, *Giardia lamblia*, *Cryptosporidium* spp. *Blastocystis hominis*, and *Dientamoeba fragilis*. In 42 of 47 faecal samples with parasites and in 44 of 44 negative samples there was agreement between microscopy and the Seegene parasite panel, Table 5.

Table 5 Performance of Seegene RT-PCR parasite panel compared with microscopy and in-house RT-PCR for protozoans

	Positive samples (47*)		Negative samples (44)	
Protozoa	Microscopy	Parasite panel	Microscopy	Parasite panel
Giardia lamblia	20	18 + 2**	0	0
Entamoeba histolytica	2	1 + 2**	0	0
Cryptosporidium spp.	8	7 + 2**	0	0
Cyclospora cayetanensis	2	2 + 0**	0	0
Blastocystis hominis	ND	45	ND	20
Dientamobia fragilis	ND	17	ND	14

ND = not done. *B. homonis* and *D. fragilis* were not recorded by microscopy as they are regarded as non-pathogenic.
* 15 of 47 positive samples were negative for the tested protozoans and 14 were negative by Seegene.
** First number was found by microscopy. Second number was only found by Seegene and could not be confirmed by the BD max parasite inhouse test.

3.4 Sensitivity and specificity of the Seegene panels.

The sensitivity for pathogenic bacteria, pathogenic *E. coli* and parasites were 95%, 100% and 89% respectively. The specificity for pathogenic bacteria, pathogenic *E. coli* and parasites were 74%, 46% and 100% respectively.

Table 6 Sensitivity and specificity of the Seegene assay

Sample	Culture/microscopy No of samples	Seegene PCR No of pos. samples	Sensitivity	Specificity
Path. bacteria pos.	19	18	95%	74%
Path. bacteria neg.	72	19		
Path. *E. coli* pos.	44	44	100%	46%
Path. *E. coli* neg.	91	49		
Path. protozoa pos.	47	42	89%	87%
Path. protozoa neg.	46	6		

The Seegene gastrointestinal assay detected almost all expected microorganisms and had a high sensitivity for all three panels. For pathogenic bacteria and pathogenic *E. coli* a high number of false positive samples were found which decreases the specificity markedly, whereas the specificity was high for parasites.

4. Discussion

When using molecular biological methods for detection of possible pathogenic microorganisms is it important that the method is reliable and easy to handle. The Seegene Gastrointestinal assay is one of the many different molecular biological methods available on the market.

The assay was easy to perform despite of the manual extraction with the QIAamp fast DNA stool mini kit since only one extraction was needed for each sample to detect all the included microorganisms. The assay detected almost all expected microorganisms and had a high sensitivity.

For pathogenic bacteria, all - except one - cultured microorganisms were detected by Seegene. The one missed was a *S. agona* which is not included in the Seegene assay. It is not clearly documented which *Salmonella* spp. are included in the Seegene assay which is a disadvantage. A substantial number of culture negative samples were found positive by the Seegene assay. In some cases, this could be explained by low sensitivity of the culture techniques used for routine samples, but a majority had high C(t) values indicating that small amounts of DNA had been present in the samples.

The samples may thus have contained low numbers of bacteria, which were impossible to culture. Alternatively, there might have been unspecific reactivity using the PCR causing false positive reactions. Another disadvantage with the Seegene assay is the many potential enteric pathogenic bacteria that are not included in the panels.

For pathogenic *E. coli* a high number of false positive test results were found in samples expected to be negative for pathogenic *E. coli*. Although the routine culture methods for pathogenic *E. coli* have low sensitivity, thus explaining some of the unexpected results. Only few samples could be confirmed as positive for pathogenic *E. coli* by the National Reference Laboratory. The assay includes a sufficient number of *E. coli* genes but it is a disadvantage that the assay detects *stx1/2* and does not differentiate *stx1* and *stx2* that differ in their pathogenetic potential.

For parasites, four of 32 protozoans were missed by the Seegene assay and six false positive samples were detected by Seegene PCR, which could not be confirmed by BD-Max™ PCR and did not fit with the patient records. Most critical was the failures in detecting *E. histolytica*.

The Seegene PCR needs to be modified to improve specificity and should subsequently be evaluated further on consecutive clinical faecal samples.

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References

[1] Odamaki T, Kato K, Sugahara H, Hashikura N, Takahashi S, Xiao JZ*, et al.* Age-related changes in gut microbiota composition from newborn to centenarian: a cross-sectional study. *BMC Microbiol* 2016; 16: 90.

[2] Davenport ER, Mizrahi-Man O, Michelini K, Barreiro LB, Ober C, Gilad Y. Seasonal variation in human gut microbiome composition. *PLoS One* 2014; 9: e90731.

[3] Hewison CJ, Heath CH, Ingram PR. Stool culture. *Aust Fam Physician* 2012; 41: 775-9.

[4] Ethelberg S, Olsen KE, Gerner-Smidt P, Molbak K. The significance of the number of submitted samples and patient-related factors for faecal bacterial diagnostics. *Clin Microbiol Infect* 2007; 13: 1095-9.

[5] Abubakar I, Irvine L, Aldus CF, Wyatt GM, Fordham R, Schelenz S*, et al.* A systematic review of the clinical, public health and cost-effectiveness of rapid diagnostic tests for the detection and identification of bacterial intestinal pathogens in faeces and food. *Health Technol Assess* 2007; 11: 1-216.

[6] Hanabara Y, Ueda Y. A Rapid and Simple Real-Time PCR Assay for Detecting Foodborne Pathogenic Bacteria in Human Feces. *Jpn J Infect Dis* 2016; 69: 471-476.

[7] Rintala A, Munukka E, Weintraub A, Ullberg M, Eerola E. Evaluation of a multiplex real-time PCR kit Amplidiag(R) Bacterial GE in the detection of bacterial pathogens from stool samples. *J Microbiol Methods* 2016; 128: 61-5.

[8] Iijima Y, Asako NT, Aihara M, Hayashi K. Improvement in the detection rate of diarrhoeagenic bacteria in human stool specimens by a rapid real-time PCR assay. *J Med Microbiol* 2004; 53: 617-22.

[9] Spina A, Kerr KG, Cormican M, Barbut F, Eigentler A, Zerva L*, et al.* Spectrum of enteropathogens detected by the FilmArray GI Panel in a multicentre study of community-acquired gastroenteritis. *Clin Microbiol Infect* 2015; 21: 719-28.

[10] Antikainen J, Kantele A, Pakkanen SH, Laaveri T, Riutta J, Vaara M*, et al.* A quantitative polymerase chain reaction assay for rapid detection of 9 pathogens directly from stools of travelers with diarrhea. *Clin Gastroenterol Hepatol* 2013; 11: 1300-1307.e3.

[11] Anderson NW, Buchan BW, Ledeboer NA. Comparison of the BD MAX enteric bacterial panel to routine culture methods for detection of *Campylobacter*, enterohemorrhagic *Escherichia coli* (O157), *Salmonella*, and *Shigella* isolates in preserved stool specimens. *J Clin Microbiol* 2014; 52: 1222-4.