

Detection of common diarrhea-causing pathogens in Northern Taiwan by multiplex polymerase chain reaction

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Abstract

Conventional methods for identifying gastroenteritis pathogens are time consuming, more likely to result in a false-negative, rely on personnel with diagnostic expertise, and are dependent on the specimen status. Alternatively, molecular diagnostic methods permit the rapid, simultaneous detection of multiple pathogens with high sensitivity and specificity. The present study compared conventional methods with the Luminex xTAG Gastrointestinal Pathogen Panel (xTAG GPP) for the diagnosis of infectious gastroenteritis in northern Taiwan. From July 2015 to April 2016, 217 clinical fecal samples were collected from patients with suspected infectious gastroenteritis. All specimens were tested using conventional diagnostic techniques following physicians' orders as well as with the xTAG GPP. The multiplex polymerase chain reaction (PCR) approach detected significantly more positive samples with bacterial, viral, and/or parasitic infections as compared to conventional analysis (55.8% vs 40.1%, respectively; $P < .001$). Moreover, multiplex PCR could detect *Escherichia coli* O157, enterotoxigenic *E coli*, Shiga-like toxin-producing *E coli*, *Cryptosporidium*, and *Giardia*, which were undetectable by conventional methods. Furthermore, 48 pathogens in 23 patients (10.6%) with coinfections were identified only using the multiplex PCR approach. Of which, 82.6% were from pediatric patients. Because the detection rates using multiplex PCR are higher than conventional methods, and some pediatric pathogens could only be detected by multiplex PCR, this approach may be useful in rapidly diagnosing diarrheal disease in children and facilitating treatment initiation. Further studies are necessary to determine if multiplex PCR improves patient outcomes and reduces costs.

Abbreviations: EIA = enzyme immunoassay, ETEC = enterotoxigenic *Escherichia coli*, RT-PCR = reverse-transcription polymerase chain reaction, STEC = Shiga-like toxin-producing *E coli*, xTAG GPP = Luminex xTAG Gastrointestinal Pathogen Panel.

Keywords: coinfection, diarrhea, gastroenteritis, multiplex PCR, Taiwan

1. Introduction

Global morbidity and mortality rates of infectious gastroenteritis have continued to rise; it is the second leading cause of death in children under 5 years of age,^[1,2] accounting for 19% of child deaths globally.^[3] The World Health Organization estimates that 1.7 billion cases of diarrhea occur annually with most occurring in tropical regions.^[1,4] Moreover, diarrheal disease accounts for a

large portion of global burden (3.6% of global disability-adjusted life years).^[5] The Centers of Disease Control in Taiwan estimates that 3% to 4% of all patients seek medical attention due to diarrhea, increasing to approximately 5% to 7% in children.^[6] Although the mortality rate of diarrheal disease in Taiwan is not high, pediatric diarrhea often occurs periodically each year with a hospital admission rate of about 12%.^[7]

The gold standard for the definitive diagnosis of bacterial (eg, *Salmonella* spp, *Shigella* spp, *Campylobacter jejuni*, and *Clostridium difficile* toxin A/B) or viral (eg, norovirus and rotavirus) infection currently depends on cultivation of the pathogenic organisms, which requires 2 to 3 days for cultivation and identification, biochemical identification of bacterial genera, or cell culture techniques.^[8,9] The probability of cultivating a pathogenic organism is greatly reduced if the specimen is not freshly collected, if the patient has used antibiotics, or due to inappropriate sample collection. Parasitic infections (eg, *Cryptosporidium* and *Entamoeba histolytica*) are commonly diagnosed by Ziehl-Neelsen staining, enzyme immunoassay (EIA), or counting every visible unstained trophozoite of *E histolytica*. However, the sensitivity of microscopic examination is low, and it relies on an experienced technologist. Although the sensitivity and specificity of EIA are acceptable, only 1 pathogen can be tested at a time. As for viruses, both EIA and polymerase chain reaction (PCR) are employed for individual detection. However, these approaches are not useful for patients without apparent pathogen-specific symptoms or those with uncommon infections or multiple infections (eg, coinfection of *Rotavirus* and *Norovirus*).

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Because early identification of the infectious agent causing diarrhea is essential to clinical practice, especially for young children and elderly patients, a comprehensive method for the identification of a diarrhea-causing pathogens, including bacterial, viral, and parasitic pathogens, is necessary. For some pathogens, molecular methods may increase detection as compared to nonmolecular methods.^[10,11] Multiplex PCR is a method that uses multiplex molecular panels for the simultaneous, rapid detection of up to 20 different pathogens pathogenic organisms.^[12] A recent meta-analysis that included 10 studies found that the gastrointestinal pathogen panels had higher positive findings as compared to conventional methods.^[13] Cost reductions have also been reported using the Luminex xTAG Gastrointestinal Pathogen Panel (xTAG GPP).^[14] The broad diagnostic capabilities of gastrointestinal pathogen panels are also particularly useful for identifying the etiological cause of diarrhea in travelers returning from tropical regions.^[15]

Because there have been no reports using this technique to evaluate infectious gastroenteritis in Taiwan, the objective of this study was to test the hypothesis that the Luminex xTAG GPP (sensitivity of 94.3% and specificity of 98.5%)^[16] has greater clinical performance in the diagnosis of infectious gastroenteritis in northern Taiwan as compared with conventional methods. We show that the detection rates using this approach are higher than the conventional method, and samples containing multiple pathogens can be identified. Furthermore, there are some pathogens, especially in children, which cannot be detected by conventional methods but are positive by multiplex PCR.

2. Materials and methods

2.1. Sample collection

From July 2015 to April 2016, a total of 217 clinical fecal samples were collected from patients with suspected gastrointestinal pathogen infections, including 124 specimens from adults and 93 from children. The Chang Gung Memorial Hospital Institutional Review Board approved the collection of the delinked samples for the present study.

2.2. Conventional diagnostic techniques

The traditional diagnostic processes include culture, microscopic examination, or detection of enteric pathogens in the stool antigen test, including a variety of steps, the choice of media, biochemical identification, viral culture, classification of serum, and analysis of drug resistance. The blood agar plate/eosin methylene blue plate (Becton Dickinson, Franklin Lakes, NJ), xylose-lysine-deoxycholate agar, and gram negative broth were purchased from Creative Microbiologicals Product (Taipei County, Taiwan) and used for the cultivation of *Salmonella* spp and *Shigella* spp. *Campylobacter* isolated agar (Creative Microbiologicals Product) was used for the cultivation of *C jejuni*. *Clostridium difficile* selective agar was used for the cultivation of *C difficile*. Thiosulfate citrate bile salt sucrose agar (Becton Dickinson) was selected for cultivation of *Vibrio cholerae*. *Escherichia coli* O157 was identified by using 16sRNA sequencing. Norovirus GI/GII and rotavirus A were identified using the LightMix Modular Gastroenteritis Assays (Roche Molecular Diagnostics, Basel, Switzerland). Parasitic pathogens, such as *Cryptosporidium*, *E histolytica*, and *Giardia*, were tested using EIAs by the ProSpect *E histolytica* Microplate Assay Procedure Card (Thermo Fisher Scientific Inc, Waltham, MA)

and real-time PCR using the LightCycler System (Roche Molecular Diagnostics). *Clostridium difficile* toxin A/B was detected by Xpert *C difficile* (Cepheid, Sunnyvale, CA).

2.3. Multiplex PCR sample processing and data analysis

Multiple reverse-transcription PCR (RT-PCR) analysis with the xTAG GPP was used for the detection of 15 types of diarrheal pathogens following the manufacturer's protocol. The panel includes adenovirus 40/41, rotavirus A, norovirus GI/GII, *Salmonella* spp, *Campylobacter* spp (*C jejuni*, *C coli*, and *C lari*), *Shigella* spp (*S boydii*, *S sonnei*, *S flexneri*, and *S dysenteriae*), *C difficile* toxin A/B, enterotoxigenic *E coli* (ETEC) LT/ST, *E coli* O157, Shiga-like toxin-producing *E coli* (STEC) stx1/stx2, *Yersinia enterocolitica*, *V cholerae*, *Giardia lamblia*, *E histolytica*, and *Cryptosporidium* spp (*C parvum* and *C hominis*). Briefly, 1 mL of NucliSENS easyMAG Lysis Buffer, 10 μ L of bacteriophage MS2, and 100 to 150 mg of feces were added to a SK38 tube that was vortexed for 5 minutes and allowed to stand at room temperature for 10 to 15 minutes prior to centrifugation at 14,000 rpm. A QIAamp MinElute Virus Spin kit (Qiagen Inc, Valencia, CA) was used for extraction of nucleic acids. An internal control sample (phage MS2) was added to each test sample prior to extraction. Multiple primers were labeled with different dyes via magnetic beads. A red LED filter with wavelength 635 nm was used to distinguish different types of beads, and a 525-nm green LED filter was used to identify those with a fluorescent response. Luminex xPONENT 4.2 software (Luminex Corp, Austin, TX) was used to acquire and analyze the data.

2.4. Statistical analysis

The frequency and percentage were calculated for all data.

3. Results

3.1. Pathogens identified by conventional analysis vs multiplex PCR

A total of 217 specimens were collected from 83 females and 134 males aged between 0.1 and 101.4 years. Analysis of each of the specimens by both conventional methods and multiplex PCR revealed that the multiplex PCR approach detected significantly more positive samples with bacterial, viral, and/or parasitic infections. Specifically, 87 (40.1%) were positive by conventional analysis, and 121 (55.8%) were multiplex PCR-positive ($P < .001$; Table 1). Moreover, 23 samples coinfecting with 48 pathogens were detected by multiplex-PCR alone. In addition, multiplex PCR could detect some pathogens, including *E coli* O157, ETEC, STEC, *Cryptosporidium*, and *Giardia*, which were undetectable by conventional methods (Table 1).

3.2. Analysis of pathogens not detected by conventional analysis vs multiplex PCR

One drawback to conventional approaches in identifying pathogens in clinical specimens is that their testing must be individually requisitioned by the treating physician based upon the patient's presentation and history. Multiplex PCR analysis does not require such individual requisitions. In the present study, 48 of 130 culture-negative samples (from 45 individuals) were positively detected by multiplex PCR (34.6%). Of the 48

Table 1
Detection of pathogens by conventional culture with PCR and multiplex PCR.

Pathogen	Conventional analysis* (n=217)	Multiplex PCR (n=217)
Detection		
Negative	130 (59.9%)	96 (44.0%)
Positive	87 (40.1%)	121 (55.8%)
Bacteria†		
<i>Campylobacter</i>	12 (5.5%)	17 (7.8%)
<i>Clostridium difficile</i> toxin A/B	21 (9.7%)	24 (11.1%)
ETEC LT/ST	Not available	1 (0.5%)‡
STEC stx1/stx2	Not available	1 (0.5%)‡
<i>E coli</i> O157	Not available	2 (0.9%)
<i>Salmonella</i> spp	27 (12.4%)	33 (15.2%)
<i>Shigella</i> spp	1 (0.5%)	3 (1.4%)
<i>Vibrio cholerae</i>	Not available	Not available
<i>Yersinia enterocolitica</i>	1 (0.5%)	1 (0.5%)
Viruses†		
Norovirus GI/GII	15 (6.9%)	28 (12.9%)
Rotavirus A	9 (4.1%)	10 (4.6%)
Adenovirus 40/41	Not available	Not available
Parasites†		
<i>Cryptosporidium</i>	Not available	1 (0.5%)
<i>Entamoeba histolytica</i>	1 (0.5%)	1 (0.5%)
<i>Giardia</i>	Not available	2 (0.9%)

ETEC=Enterotoxigenic *Escherichia coli*, PCR=polymerase chain reaction, STEC=Shiga-like toxin-producing *E coli*.

* Conventional analysis includes the bacterial/viral culture methods with single PCR.

† Only positive detection data were used for analysis.

‡ Coinfections detected by multiplex PCR.

pathogens that were identified using the multiplex PCR approach, 32 (66.7%) of them were not requested to be examined by the ordering physician; 16 samples had no growth using conventional methods (Table 2).

3.3. Analysis of pathogens implicated in coinfections

The majority of pathogens tested in our hospital are typically requisitioned 1 pathogen at a time, resulting in almost no chance

of detect coinfection by conventional analysis. In contrast, 19% (23/121) of the specimens had been confirmed as having coinfection using multiplex PCR analysis (Table 3). Most specimens with coinfections were from children; the top 3 detected by multiplex PCR were *C difficile*, *Salmonella* spp, and norovirus. In elderly patients, *C difficile* and Norovirus GI/GII species were the main pathogens in coinfections.

4. Discussion

In this study, 217 fecal specimens of adult and pediatric patients with acute diarrhea were examined by both conventional methods, including culture and PCR, according to the physician's order for detection of suspicious pathogens and multiplex PCR. We found that the multiplex PCR approach detected significantly more positive samples, including some that were undetectable by conventional methods (eg, *E coli* O157, ETEC, STEC, *Cryptosporidium*, and *Giardia*). A greater number of coinfections were also identified by multiplex PCR. Finally, 19% of pathogens identified by multiplex PCR were not requested to be examined by the ordering physician, highlighting the difficulty in predicting a specific etiology and preselecting appropriate assays for pathogen detection.

At present, traditional methods for identifying bacterial, viral, and parasitic infections have the following drawbacks: time-consuming, restricted by methodologic limitations, more likely to result in false-negative results, and rely on personnel with diagnostic expertise and status of the specimens. In contrast, multiplex PCR only requires 3.5 hours and can test for the presence of multiple pathogens at the same time with high accuracy. Also, multiplex PCR assays are sensitive enough that stool specimens may be screened directly without cultivation.^[17,18] In the present study, the positive rate for pathogens using conventional culture or PCR methods was 40.1%, whereas the positive rate of multiplex PCR was significantly higher at 55.8%. This increase in detection is consistent with a previous study in which real-time PCR panels were employed to detect viral-induced diarrheal disease^[19] as well as other studies in which a xTAG GPP had equivalent or increased sensitivity as compared to conventional methods.^[20,21] This also is consistent

Table 2
Pathogens detected by multiplex PCR, but not detected by conventional analysis, including samples with no growth and those without requisition.

Target	Total no of multiplex detected	Conventional analysis			Total n (%)
		No growth	Non-requisition		
Negative	96	0	0	–	
STEC	1	–	0	–	
<i>Yersinia enterocolitica</i>	1	0	0	–	
ETEC	2	–	0	–	
<i>Entamoeba histolytica</i>	1	0	0	–	
<i>Salmonella</i> spp	44	7	1	8 (18.2)	
<i>Campylobacter</i>	18	4	1	5 (27.8)	
<i>Giardia</i>	2	2	0	2 (100)	
<i>Shigella</i> spp	3	2	0	2 (66.7)	
<i>Clostridium difficile</i> toxin A/B	38	1	14	15 (39.5)	
Norovirus GI/GII	30	0	11	11 (36.7)	
Rotavirus	11	0	3	3 (27.3)	
<i>Cryptosporidium</i>	1	0	1	1 (100)	
<i>Escherichia coli</i> O157	2	–	1	1 (50)	
Total	250	16	32	48 (19.2)	

Dash indicates no data. Data presented were the row percentages.

ETEC=Enterotoxigenic *Escherichia coli*, PCR=polymerase chain reaction, STEC=Shiga-like toxin-producing *E coli*.

Table 3**Detailed analysis of coinfections detected by multiplex PCR.**

Specimens source	Number of cases (n = 23)	Conventional analysis	Additional pathogens detected by multiplex PCR	
Children	1	<i>Salmonella</i> spp	<i>Clostridium difficile</i> toxin A/B, ETEC LT/ST STEC stx1/stx2	
	4	<i>Salmonella</i> spp	<i>C difficile</i> toxin A/B	
	1	<i>C difficile</i> toxin A/B	Norovirus GI/GII, <i>Salmonella</i> spp	
	1*	Norovirus GI/GII, rotavirus A*	Norovirus GI/GII, rotavirus A	
	1	Norovirus GI/GII	<i>Salmonella</i> spp, <i>C difficile</i> toxin A/B	
	1	Norovirus GI/GII	<i>Salmonella</i> spp, ETEC LT/ST	
	2	Norovirus GI/GII	<i>Salmonella</i> spp	
	1	Norovirus GI/GII	<i>Salmonella</i> spp, <i>Campylobacter</i>	
	4	Norovirus GI/GII	<i>Salmonella</i> spp, <i>C difficile</i> toxin A/B	
	1	Negative	Norovirus GI/GII, <i>C difficile</i> toxin A/B	
	1	Negative	<i>Salmonella</i> spp, <i>C difficile</i> toxin A/B	
	1	Negative	Norovirus, <i>Salmonella</i> spp	
	Adults/elderly†	1	<i>Salmonella</i> spp	<i>C difficile</i> toxin A/B, <i>E coli</i> O157
		1	<i>C difficile</i> toxin A/B	<i>Salmonella</i> spp
1		<i>C difficile</i> toxin A/B	Norovirus GI/GII	
1		Negative	Norovirus GI/GII, <i>C difficile</i> toxin A/B	

ETEC=Enterotoxigenic *Escherichia coli*, PCR=polymerase chain reaction, STEC=Shiga-like toxin-producing *E coli*.

* The physician made 2 requisitions for clinical microbiology tests.

with Mengelle et al^[22] in which the xTAG GPP was more sensitive than conventional tests at detecting rotavirus, norovirus, *Salmonella* spp, *Campylobacter* spp, and *C difficile*.

A previous analysis of the xTAG GPP showed that testing of a particular pathogen was not ordered by the physician in 65% of the specimens.^[16] In Taiwan, physicians rarely order testing for more than one type of pathogen in clinical practice despite that approximately 19% of the samples in the present study had coinfections. Multiplex PCR was better at identifying the presence of coinfections, which is often difficult using a single conventional method. This is particularly important given that 83% of the specimens from pediatric patients were found to have coinfections. The increased detection of coinfections with the xTAG GPP as compared to conventional methods is similar to that reported by Deng et al^[23] in detecting enteropathogens in Southern China.

In the present study, *Salmonella* spp and *C difficile* toxin A/B were identified as the main causes of diarrhea; they were also implicated in coinfections in both children and patients ≥ 60 years of age. However, norovirus GI/GII was found in 72.2% of the samples that had coinfection in children, which is consistent with previous studies that reported high rates of norovirus coinfections.^[24]

Previous studies indicate that EIA kits for detecting *Giardia* and *Cryptosporidium* have low specificity and sensitivity and should not be used as the only diagnostic test in low-prevalence areas.^[25] Multiplex PCR analysis was also able to detect parasitic infections that are often overlooked in Taiwan. Specifically, *Cryptosporidium* and *Giardia* were detected in 0.5% and 0.8% of the samples, respectively; however, neither could be detected by conventional methods.

The present study has limitations that warrant discussion. Although we showed that multiplex PCR was better than conventional methods at detecting pathogens as well as coinfections, which may result in faster diagnosis and better disease management, patient outcomes and health economic impact were not assessed. Additionally, the panel itself was not tested to examine whether there were pathogens in the specimens that were not included in the 15-pathogen panel. Thus, further studies will examine whether addition of other diarrhea-causing pathogens, such as *Dientamoeba fragilis*,^[26] would have clinical

benefit. Furthermore, as opposed to chromogenic culture methods, multiplex PCR analysis does not provide information regarding antibiotic susceptibility or may not detect new genes or gene variants.^[27]

In conclusion, xTAG GPP significantly shortens the time required for testing and may provide some benefit for the treatment or management of infectious gastroenteritis patients by providing a more rapid, accurate diagnostic result even for those with coinfections. This aspect is particularly important given that 83% of the specimens from the pediatric patients showed coinfection. Furthermore, this panel successfully identified parasitic infections, which are often overlooked sources of diarrheal disease in Taiwan. Further studies are necessary to determine if diagnosis of gastroenteritis by multiplex PCR results in improved patient outcomes and reduced costs.

Author contributions

Shu-Huan Huang: guarantor of integrity of the entire study; study concepts; study design; definition of intellectual content; clinical studies; experimental studies; data analysis; manuscript preparation.

Yi-Fang Lin: experimental studies; data acquisition; statistical analysis.

Ming-Han Tsai: study design; definition of intellectual content.

Shuan Yang: clinical studies; data acquisition.

Mei-Ling Liao: clinical studies; data acquisition.

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