The application and epidemiological research of xTAG-GPP multiplex PCR in the Diagnosis of children persistent and chronic diarrhea

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Chunli Wang
Children's hospital of Nanjing Medical University

Xiaoying Zhou
Children's hospital of Nanjing Medical University

Mengshu Zhu
Children's hospital of Nanjing Medical University

Hanjun Yin
Children's hospital of Nanjing Medical University

Jiamei Tang
Children's hospital of Nanjing Medical University

Yan Huang
Children's hospital of Nanjing Medical University

Bixia Zheng
Children's hospital of Nanjing Medical University

Yu Jin
Children's hospital of Nanjing Medical University

Zhifeng Liu
Children's Hospital of Nanjing Medical University

etyygdz@163.com*Corresponding Author*

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Abstract

Background Persistent and chronic diarrhea is difficult to treat, and infection is still the main causes. Clearly infected pathogens are essential for treatment. In this study, we investigate the application value of xTAG gastrointestinal pathogen panel (xTAG GPP) multiplex PCR in the early diagnosis of children with persistent and chronic diarrhea and understand the epidemiology of intestinal diarrhea pathogens.

Methods One hundred and ninety-nine specimens were collected from Nanjing Children's Hospital Affiliated to Nanjing Medical University (Nanjing, China). Comparing the xTAG GPP multiplex PCR Assay with the traditional methods (culture, rapid enzyme immunoassay chromatography, microscopic examination) and madding the statistical analysis.

Results The positive rate of 199 patients with diarrhea specimens was 72.86% (145/199). The virus detection rate was 48.7%, Rotavirus A was the most common organism detected (34.6%), concentrated in winter, popular in children. Secondly, Norovirus GI/GII (20.6%). The positive rate of bacteria was 40.2%, Campylobacter (22.11%, 44/199) was most frequently detected. With C. difficile toxins A/B and Salmonella detected 44 and 17 samples, respectively. Infections with Shigella occurred 4 times, E. coli O157 was only detected once. There were three samples with parasitic (1.51%), two samples were positive for Entamoeba histolytica, one for Cryptosporidium. Adenovirus40/41, STEC, ETEC, Giardia, Yersinia enterocolitica and Vibriocholera were not detected. Totally 86 (43.2%) infected specimens with single pathogen were detected. There were 57 co-infections (28.64% of samples) of viruses and/or bacteria and/or parasites. Co-infections involved 29 double infections (23.62%) samples, 9 triple infections (4.52%) and 2 quadruple infections (0.5%). Norovirus GI/GII was found to have the highest involvement in co-infections 30(15.08%).

Conclusion xTAG GPP multiplex PCR is simple, sensitive, specific and can be used as a quick way to diagnose the children persistent and chronic diarrhea.

Background

Diarrhea continues to be a health burden world-wide, especially in children living in developing countries. It is estimated that in these regions it is responsible for 2.5 million infant deaths annually,

with a mortality rate of 4.9 per 1,000 children and anannual incidence of 3 episodes per child among children under 5 years of age [1, 2]. Most of the diarrheal illnesses are acute, lasting no more than 7 days; however, about 3%-19% of the acute episodes lasting more than two weeks, which called persistent and chronic diarrhea [3]. Persistent and chronic diarrhea cases are difficult to treat, their treatment cost is higher, and a case fatality rate as high as 60% has been reported [4]. It is more important to determine the cause of chronic diarrhea by a systematic approach, because it can provide the most suitable therapy and give a good prognosis. The causes of chronic diarrhea are divided into infectious and non-infectious causes. In the developed countries, the incidence of noninfectious based diseases (food allergy, enteropathy or inflammatory) is increasing. However, in developing and industrialized countries, the most common and most important cause of persistent and chronic diarrhoea is still enteric infection [5, 6]. It is crucial for timely and effective treatment of infectious diarrhea in the rapid identification of pathogens, because appropriate antimicrobial therapy and/or isolation measures to prevent the spread of infectious patients from healthy people can shorten the disease and reduce some bacteria and parasites infection incidence, and can help the invasive infection [7, 8]. The gold standard for the diagnosis of infectious chronic diarrhea pathogens is the culture of pathogens, but this method takes a long time (72 hours) and requires higher fecal samples[9]. In recent years, the development of faster and more-sensitive molecular tests that can detect various pathogenic agents of bacteria, viruses and parasites might improve the etiological diagnosis of diarrhea pathogens [9–11].

The Luminex® Corporation has developed a new qualitative bead-based multiplexed molecular diagnostic test, the xTAG Gastrointestinal Pathogen Panel (xTAG GPP) that can be performed directly on stool samples to timely detect and identify 15 pathogens: Adenovirus 40/41, Campylobacter, Clostridium difficile, Cryptosporidium, Entamoeba histolytica, Enterotoxigenic Escherichia coli (ETEC), E. coli O157, Shiga-like Toxin producing E.coli (STEC), Shigella, Salmonella, Giardia, Norovirus GI/GII, Rotavirus A, Vibriocholerae and Yersinia enterocolitica [12]. The clinical manifestation of xTAG GPP were recently evaluated in many infectious gastroenteritis, the GPP have more sensitivity and specificity than traditional methods [13, 14].

Thus, the purpose of our study was to explore the distribution of enteropathogens in persistent and chronic diarrheal patients in Nanjing, China, and to further evaluate the performance and applicability of xTAG GPP in identifying pathogens in these children.

Methods

Sample collection

A total of 199 stool samples prospectively collected from 199 diarrhoeic children mainly under 5 years of age (85.93%, Table 1) which including 103 simple of diarrhea and 88 secondary diarrhea (colitis, pneumonia and tumor associated) that attending the Nanjing Children's Hospital Affiliated to Nanjing Medical University (Nanjing, China). The study protocol was approved by the ethics committee of the Children’s Hospital of Nanjing Medical University (Nanjing, China). Written informed consent was obtained from the proband and their parents. One sample was received from each patient. Inclusion criteria□patients with diarrhoeic present watery and/or loose and/or mucous and/or blood stools with ≥3 instances within a 24-hour period. Patients with inflammatory bowel diseases were excluded from the study. Stool samples were sent to the Department of Microbiology for investigation. 5g of fresh stool samples were collected into empty tube were placed in Carye Blair transport Medium for bacterial culture. Stool specimens were then stored at -80°C until processing with the multiplex PCR tests. Unqualified samples (samples volume < 5g, swabs not preserved in Carye Blair Medium) were rejected and resubmission requested.

Routine diagnostic methods

Stool culture for Salmonella and Shigella was performed using Salmonella–Shigella agar plates and Hektoen enteric agar plates. To detect toxigenic Clostridium difficile A and B toxins, norovirus GI/GII, realtime reverse transcription-polymerase chain reaction (RT-PCR) assays were performed on the 7500 real-time PCR platform (Applied Biosystems, Foster City, CA). Rotavirus were detected directly in stool samples with rapid enzyme linked immunosorbent assay (ELISA) tests: Diagnostic Kit for Rotavirus. All assays were carried out in accordance with the instructions. We looked for Entamoeba histolytica and Giardialiamblia by microscopic examination of fresh stools.

Multiplex PCR assays for 15 pathogens detection/Molecular diagnostic assay

Total nucleic acids were extracted from the stool samples using the NucleoSpin® Virus Kit (MACHEREY-NAGEL, Germany) according to the manufacturer's instructions. An internal control (bacteriophage MS2) was included in each specimen to control the quality of the detection process. The RT-PCR reactions and subsequent hybridization step were performed according to the instructions in the GPP manual. Negative and positive controls were included in all runs of the GPP assay. The data were acquired on the Luminex 200 analyzer and data analysis was carried out using TDAS GPP version 1.11 (xTAG Data Analysis Software).

Results

Demographic and clinical parameters of patients with persistent and chronic diarrhea

The demographic and clinical characteristics of the 199 patients are summarized in Table 1. One hundred and ninety-nine stool samples were prospectively collected from 199 diarrhoeic children mainly under 5 years (85.93%, 171/199), mean age was 12.93±15.86 months. The percent of boys (58.29%, 116/199) was slightly higher in comparison to girls (41.71%, 83/199). There were 163 persistent diarrhea cases and 28 chronic case, the majority were the inpatients (88.44%, 176/199) during the study period, no deaths were reported. Of the 199 stool specimens submitted to laboratories, watery/loose stool (n=139, 72.78%) were the most common type, mucus/bloody stool were less about 27.22% (52/199).

Pathogens detected with the xTAG GPP

In this study, we found 145 (72.86%) positive samples collected from 199 patients. Of these, 97 samples were positive for viruses, with rotavirus A being the most common organism detected (34.67%; 69/199), the second abundant virus was norovirus GI/GII, which was detected in 41 cases (20.6%; 41/199). Bacterial pathogens accounted for 40.2% (80/199) of all enteropathogens, Campylobacter (22.11%□44/199) was most frequently detected, with C. difficile toxins A/B and Salmonella detected 44 and 17 samples, respectively. Infections with Shigella occurred 4 times, E.

coli O157 was only detected once. There were three samples with parasitic(1.51%)，two samples were positive for Entamoeba histolytica，one for Cryptosporidium. Adenovirus 40/41, STEC, ETEC, Giardia, Yersinia enterocolitica and Vibriocholera were not detected. There were 57 co-infections (28.64% of samples) of viruses and/or bacteria and/or parasites (Table 2). Co-infections involved 29 double infections (23.62%) samples, 9 triple infections (4.52%) and 2 quadruple infections (0.5%). Norovirus GI/GII was found to have the highest involvement in co-infections 30 (15.08%), followed were Rotavirus A (14.8%, 29/199), Campylobacter (13.29%, 23/199) and Clostridium C.difficile Toxin A/B (9.05%, 18/199) (Table 4).

Comparison of the xTAG GPP and conventional detection

Among the enteropathogens that can be detected by xTAG GPP, 5 enteropathogens cannot be detected by routine detection(STEC, ETEC, Adenovirus 40/41, Yersinia enterocolitica and Campylobacter), so in this study, the specificity and sensitivity of this method for the diagnosis of these five enteropathogens are not compared. As shown in Table 4, the sensitivity was 100% for Norovirus GI/GII, C. difficile toxin B and Shigella, 96.9% for rotavirus A and 33.3% for Salmonella. The specificity was 100% for all targets except Entamoeba histolytica (99.5%), E. coli O157 (99.0%), Cryptosporidium (99.0%), Shigella (98.0%), Salmonella (92.3%), Rotavirus A (89.3%), Norovirus GII (89.3%) and C. difficile toxin A/B (84.9%). Among the 10 comparable enteropathogens, 2 enteropathogens（Giardia and Vibriocholera） have not been detected positive samples in our samples by both xTAG GPP and the routine assays, so it is impossible to evaluate the sensitivity of these enteropathogens. The overall sensitivity and specificity of xTAG GPP for the diagnosis of intestinal pathogens were 96.3% and 98.2% respectively, which were significantly higher than those of conventional detection. The sensitivity and specificity of this method to individual pathogens are shown in Table 4.

Age and sex distribution of children with enteropathogens

The prevalence of enteropathogens among sex groups was compared, 75.86% male patients and 57

(68.67%) female patients were positive for enteropathogens. The distribution of enteropathogens was similar in both boys and girls (Table 5), with Rotavirusas A the most common pathogen detected at 39.66% and 27.71%, respectively, followed by Campylobacter and Norovirus GI/GII, there was also no significance in co-infection ($p>0.05$). The distribution of virus, parasite and co-infection were similar in the three age group (0-12months, 12-60 months and ≥60 months), the *P* value were 0.73, 0.724 and 0.76 respectively (Table 5). Rotavirus A was the most common enteropathogen in patients 0-12 months (37.9%,) and 12-60 months (33.33%) in age, while Campylobacter was the most frequent enteropthogen in patients ≥60 months (28.6%, 8/18). In this study, Bacteria infections were the most common in the 12-60 months age group (57.1%) compared with the other age groups (33.3%-46.4%).

Seasonal distribution of children with enteropathogens

In this study, the seasonal curve of virus had a peak in winter and trough in summer, Rotavirus A was the most important enteropathogens, the infection peak occurred in the November 2014 to February 2015 (Fig 1), with the highest proportion occurring in December 2014 (90.0%, 18/20). In contrast, bacterial agents had a peak in summer and trough in winter, Campylobacter was the most frequent enteropthogen and the highest proportion occurring in October 2015 (75.0%, 15/20).

Discussion

There are few data that simultaneously describe the prevalence of bacterial and viral pathogens in persistent and chronic diarrhea children in China. In our research, the persistent and chronic diarrhea have related to the sex and age, common exist in the boy especially under 2 years old children, in our study, the boy was 116 (58.29%), 0–1 years patient was 129 (64.82%), this was similar with the research that the morbidity age was 4 months to 1 year [3]. Patient with watery and/or loose were predominate more than the mucoid/bloody group consider that the most sample were collected in winter (Table 4).

In this study, the overall sensitivity and specificity of, xTAG GPP were 96.3% and 98.2% respectively, which were significantly different from those of routine detection. At the same time there were

significant differences in single or mixed intestinal enteropathogen infection ($P < 0.001$) (Table 4). In the present study, the xTAG GPP method efficiently detected about 57(28.64%) out of 199 children showed multiple positive results (co-infection) (Table 2), this figure is higher than that was relatively high compared with the positive result also detected by xTAG GPP in Deng J et al previous study [13]. Norovirus GI/GII was found to have the highest involvement in co-infections in our study.

In our study, Rotavirus A was the most common pathogen in children with chronic diarrhea in spring and autumn, followed by Norovirus. Previous reports have shown that Rotavirus A is the most common virus that causes diarrhea in children[15]. Moreover, Norovirus is an important cause of diarrhea in adults and children [16].

This result was similar to other studies conducted previously in China [17–19] as well as other countries prior to the introduction of rotavirus vaccination [20, 21].

In our study, 4 samples with positive Salmonella culture had no positive results in xTAG GPP, existing false negative results. This phenomen is consistent with some previously reported results [13, 22]. The cause of the failure for Salmonella pathogen needs further sequence analysis or qPCR assay investigation. Special attention should be paid to the occurrence of Campylobacter, in our study we detected a high frequency of 25% (43/199) by the xTAG GPP assay, because in China the detection of Campylobacter is rarely requested in patients with diarrhea.

Conclusions

In conclusion, our research shows that xTAG GPP has very good sensitivity and specificity in detecting pathogens associated with persistent and chronic diarrhea. This method can shorten the detection time and reduce the false negative diagnostics, identify the cause of infection more quickly and accurately, provide a basis for follow-up clinical accurate treatment and improve the prognosis of the disease. However, the number of samples in this experiment is limited, some pathogens have no positive samples(Giardia and Vibriocholera), so it is impossible to compare.

Abbreviations

xTAG GPP

xTAG gastrointestinal pathogen panel

ETEC

Enterotoxigenic Escherichia coli

STEC

Shiga-like Toxin producing E.coli

RT-PCR

realtime reverse transcription-polymerase chain reaction

Declarations

Ethics approval and consent to participate

The study protocol was approved by the IEC of the Children's Hospital of Nanjing Medical University (Nanjing, China) and approval number is 201901013-1. Informed consent, additional clinical information and stool samples were obtained from all subjects. The study protocol was approved by the ethics committee of the Children's Hospital of Nanjing Medical University (Nanjing, China). Written informed consent was obtained from the proband and their parents.

Consent for publication Not applicable

Availability of data and materials

All data generated or analysed during this study are included in this published article.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

CLW and XYZ researched the topic, analyzed the data, and was a major contributor in writing the manuscript. MSZ and XYW collected clinical stool samples and clinical data. HJY, YH and JMT completed relevant experiments and collated experimental data. ZFL, YJ and BXZ guided writing and critically reviewed the manuscript. All authors read and approved the final manuscript.

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Tables

Table 1. Demographic and Clinical characteristics of the study subjects.

Characteristics	No (%)
Demographics	
Boys	116(58.29)
Girls	83(41.71)
Age (year)	
0-1	129 (64.82)
1-5	42(21.11)
≥5	28(14.6)
Patients	
Out-patients	23(88.44)
In-patients	176(11.56)
Course of disease (week)	
2-8	163(85.34)
≥8	28(14.66)
Appearance of diarrhea	
watery /loose	139 (72.78)
Mucoid / bloody	52(27.22)
Defecation frequency (Times / day)	
3-5	69(36.13)
5-10	82(42.93)
≥10	40(20.94)
Use of antibiotics	
Used	151(79.06)
Unused	40(20.94)
Stool culture	
not done	42(21.99)
negative	114(59.69)
positive	35(18.32)
Diarrhea type	

Simple diarrhea	103(53.93)
Secondary diarrhea	88(46.07)

Table2. Numbers of single and multiple infections detected by xTAG GPP

Infection	Number	No. (%)
Single	86	43.21%
Double	47	23.62%
Triple	9	4.52%
Quadruple	1	0.5%
Multiple	57	28.64%

Table 3. Pathogens detected in co-infections

pathogen	Double infection	Triple infection	Quadruple infection	Co-infection
Rotavirus A	21	7	1	29(14.58%)
Norovirus GI/GII	25	4	1	30(15.08%)
Clostridium difficile Toxin A/B	12	5	1	18(9.05%)
Salmonella	8	2	0	10(5.03%)
Campylotrbacter	19	4	0	23(13.29%)
Shigella	3	1	0	4(1.73%)
Escherichia coli O157	0	1	1	2(1.16%)
Entamoeba histolytica	1	1	0	2(1.16%)
Cryptosporidium	1	0	0	1(0.58%)

Table 4. Comparison of xTAG GPP with the routine tests and the results of XTAG GPP for the detection of enteric pathogens from patients with persistent and chronic diarrhea.

Class	Target	GPP	No. of samples by routine tests		Performance of the xTAG G	
			+	-	%Sensitivity	%Specif
Virus	Adenovirus 40/41	+	0	0		
		-	0	199	0	1
	Rotavirus A	+	63	6		
		-	2	50	96.9	8
	Norovirus GI/GII	+	22	19		
		-	0	158	100	8
Bacteria	*Salmonella*	+	2	15		
		-	4	182	33.3	9
	Campylobacter	+	43	0		
		-	0	199	100	1
	Shigella	+	1	3		
		-	0	195	100	9
	*Clostridiumdifficile*Toxin A/B	+	27	3		
		-	0	169	100	8
	ETEC	+	0	0		
		-	0	199	0	1
	*Escherichiacoli*O157	+	0	2		
		-	0	197	0	9
	STEC	+	0	0		
		-	0	199	0	1
	Yersinia enterocolitica	+	0	0		
		-	0	199	0	1
	Vibrio cholerae	+	0	0		
		-	0	199	0	1
Parasite	*Giardia*	+	0	0		
		-	0	199	0	1
	Entamoeba histolytica	+	0	1		
		-	0	198	0	9
	Cryptosporidium	+	0	2		
		-	0	197	0	9
	Total	+	158	51		
		-	6	2739	96.3	9

Table 5. Age and sex distribution of children with enteropathogens

		Number	Negative	Virus	Bacteria	Parasite	Co-infection
	Boys	116	28	60	47	3	38
Sex	Girls	83	26	37	33	0	19
	χ2		1.264	0.98	0.012	2.179	2.305
	P		0.26	0.32	0.94	0.14	0.129
	0-1	129	33	70	43	2	36
Age(year)	1-5	42	11	18	24	1	14
	≥5	28	11	9	13	0	7
	χ2		2.216	5.244	7.997	0.646	0.668
	P		0.33	0.73	0.018	0.724	0.716

Figures

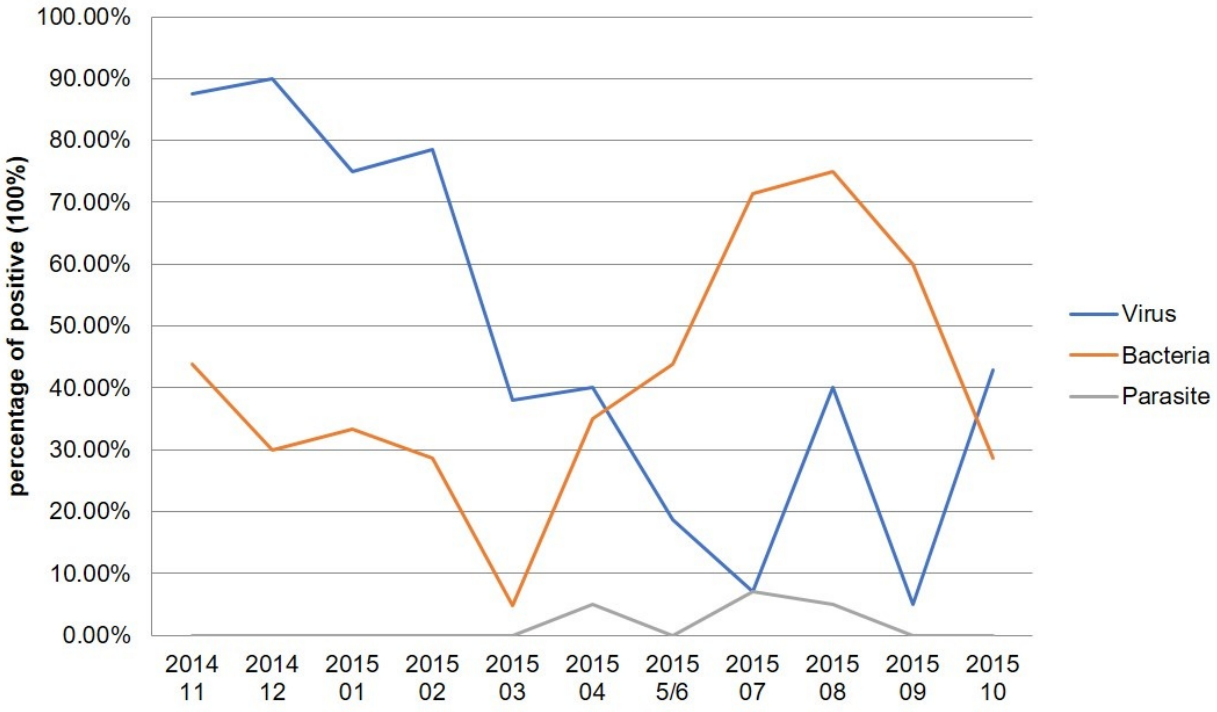

Figure 1

Seasonal distribution of children with enteropathogens detected by xTAG GPP assay.