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Original Article

Detection of viral and bacterial pathogens using multiplex real-time polymerase chain reaction in acute gastroenteritis

Melda Ozdamar[1], Berrin Uzun[2], Salih Turkoglu[1]

[1]Anadolu Medical Center, Department of Clinical Microbiology, Kocaeli, Turkey
[2]Katip Celebi University Ataturk Research and Training Hospital, Medical Microbiology Laboratory, Izmir, Turkey

Abstract

Aim: Recently, the syndromic panel-based testing approach has gained popularity in the laboratory diagnosis of gastroenteritis. This study aimed to share our experience in the use of multiplex real-time polymerase chain reaction (PCR) in acute gastroenteritis.
Material and Methods: Stool samples were obtained from 86 patients with acute gastroenteritis presenting with fever, bloody diarrhea, and dehydration in Kocaeli and İstanbul between January 2017 and November 2018. The identification was carried out using "FTD viral&bacterial gastroenteritis" (Fast Track Diagnostics, Luxembourg) kits.
Results: The causative agents were identified by multiplex PCR in 53.5% of the samples. A significant relationship was found between the age groups (16-years and older versus under 16-years) and the distribution of agents (p=0.012). A single agent was detected in 41 of 86 samples and co-infection was detected in 5 samples. The most commonly detected viral agents were *NorovirusG2, Rotavirus, Astrovirus, Adenovirus, NorovirusG1 and Sapovirus*, and for the bacterial agents were *Salmonella spp., Shigella* spp. /EIEC and *Campylobacter coli/ jejuni*, verocytotoxin-producing *Escherichia coli* (VTEC) and *Clostridium difficile* in orderly.
Conclusion: The use of molecular methods may be advised, particularly in high-risk patients. This will ensure the better evaluation of the patients and prevent inappropriate antibiotics usage, as the clinicians are able to resolve diagnostic challenges using more appropriate methods. The validation of diagnostic algorithms that prioritize the testing of certain microorganisms based tests according to their incidence especially in outpatients would seem to be an appropriate first task as a means of avoiding the application of these relatively expensive tests in all patients.

Keywords: Acute gastroenteritis; real-time polymerase chain reaction (PCR); syndromic panel-based testing; viral gastroenteritis; bacterial gastroenteritis

INTRODUCTION

Gastroenteritis is a condition characterized by mucosal inflammation of the gastrointestinal tract. Although it can affect all age groups, it is more common in the pediatric population. In some countries, gastroenteritis represents the second most common cause of death after cardiovascular disorders. In contrast with the developed countries, gastroenteritis is associated with high rates of mortality and significant socioeconomic burden in the developing world (1-3).

Gastroenteritis describes a clinical condition in which stomach and small intestine are conjointly involved, causing symptoms such as nausea, vomiting, diarrhea, and abdominal pain. Gastroenteritis may be due to infectious or non-infectious agents (1, 4). Leading infectious causative agents include bacteria such as *Shigella spp, Salmonella spp, Yersinia spp, Campylobacter spp, Aeromonas spp,* intestinal pathogenic strains of *Escherichia coli*, and *Clostridium difficile;* protozoa such as Giardia intestinalis, Entamoeba histolytica, and Cryptosporidium spp; viruses such as *Rotavirus, Norovirus*, enteric Adenovirus, enteric Coronavirus, and *Astrovirus*; as well as several fungal species (4-6). Together with advances in medicine, certain less common organisms may also be identified in patients with gastroenteritis resulting from drug side effects and/or immune suppression caused by the underlying disorders.

Bacterial cultures remain the gold standard technique for the detection of bacterial agents. Despite the generally good performance of conventional identification

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Corresponding Author: Melda Ozdamar, Anadolu Medical Center, Department of Clinical Microbiology, Kocaeli, Turkey
E-mail: melda.ozdamar@gmail.com

techniques for microorganisms growing in the culture media, these methods are also associated with certain drawbacks including the requirement for labor-intense procedures and proneness to mistakes in the selection of the diagnostic tests (7). For most viruses, it is not possible to detect the causative agent using viral cell cultures. In most instances, rapid antigen tests are utilized for routine diagnosis of these infections, due to low availability of sophisticated laboratories that can perform cell cultures as well as due to low rates of growth in cell cultures (6, 8, 9).

Among factors associated with increased morbidity, mortality, and economical burden in patients with gastroenteritis are the unreliability of clinical history and manifestations in the differential diagnosis of causative agents, presence of numerous types and numbers of causative agents, and failure of the routine laboratory tests to detect most organisms can be listed. In certain patient groups, inappropriate and sometimes indispensable use of antibiotics due to failure to detect the causative agents may not only lead to significant costs, but also to the increasingly more common selection of resistant strains causing a significant public health problem (2-10).

Rapid and correct diagnosis are not only essential but also of utmost importance in terms of the prevention/control of infectious diseases, provision of appropriate antimicrobial or anti-parasitic medications and epidemiological data analyses. Today, syndromic panel-based tests aiming at detecting the causative agents of gastroenteritis have been put into routine practice, particularly for adult and pediatric patients with immune-suppression, for subjects in whom the identification of the organism carries clinical significance due to manifestations such as high fever, bloody diarrhea, or dehydration, or in high-risk patient groups requiring hospital admission (9, 11). Molecular test panels are being increasingly available and represent highly sensitive and specific diagnostic methods that can simultaneously detect bacteria, viruses, and sometimes parasites utilizing a single reaction, obviating the need for meticulous and time-consuming conventional diagnostic methods (7, 12, 13). In 2017 IDSA (Infectious Disease Society of America) guidelines, rapid molecular test panels are positioned as diagnostic methods to be used for public health purposes in the identification of the causative agents in disease outbreaks, rather than use in individual cases. Although advanced algorithms for the clinical use of these tests are currently unavailable, their contributions to antimicrobial management programs are being continuously reported in studies across different countries and in different guidelines (13, 14).

This study was undertaken to share our experience on the routine use of multiplex RT PCR in the detection of bacterial and viral causative agents of acute gastroenteritis, to gather "modern" and up-to-date data on the epidemiology of these causative agents in our country, where such data is inadequate, and to contribute to the existing literature regarding its utility.

MATERIAL and METHODS

Stool samples obtained from a total of 86 patients from Istanbul and Kocaeli provinces admitted and hospitalized from the emergency room or outpatient clinics conservatively managed between January 2017 and November 2018 due to a diagnosis of acute gastroenteritis and presenting with fever, bloody diarrhea, and dehydration were included. The study protocol was approved by the institutional ethics committee on 01 Feb 2019 (no: ASM-Appendix 18/101). All procedures were carried out in compliance with the relevant laws and guidelines and in accordance with the ethical standards of the Declaration of Helsinki. After informed consent was obtained from patients or their legal representatives, a stool PCR panel test was requested by the treating clinician. Approximately 30 g of stool sample was collected into a clean container from each patient and was transferred to clinical microbiology laboratory within 30 minutes. After direct microscopic examination of the samples, they were stored at + 4 °C until the time of PCR analysis. Nucleic acid purification was performed using a Qiagen EZ1 Virus Mini Kit v2.0 (Qiagen, Hilden, Germany) within 12 hours on the same day of examination. Using FTD viral gastroenteritis and FTD bacterial gastroenteritis kits (Syndromic multiplex real-time polymerase chain reaction (PCR) kits; Fast Track Diagnostics, Luxembourg), nucleic acid amplification was performed according to manufacturer's instructions using a Rotor Gene Q Real-Time PCR Device (Qiagen, Hilden, Germany). After reporting of the test results to the treating clinicians, treatments were ordered and evaluated.

The following 12 potential causative agents could be examined in the same sample and test session using FTD viral gastroenteritis and FTD bacterial gastroenteritis multiplex PCR tests: *Norovirus (GI and GII), Human Adenovirus, Human Astrovirus, Rotavirus, Sapovirus, Campylobacter coli/jejuni, enterohemorrhagic Escherichia coli (EHEC), verocytotoxin-producing Escherichia coli (VTEC), Salmonella spp., Shigella/ enteroinvasive Escherichia coli (EIEC), Yersinia enterocolitica, Clostridium difficile*. Since the potential parasitic agents were not included in the test panel, these organisms were investigated using conventional methods at the discretion of the treating physician. These results have not been reported.

SPSS windows version 21 (Armonk, NY: IBP Corp.) was used for statistical analyses. Chi-square test was used to evaluate the differences between the two sexes and age groups. The level of statistical significance was set at a p value of less than 0.05.

RESULTS

Forty-five female (52.3%) and 41 male (47.7%) patients were included in this study. Among these, 47 (55%) were treated as inpatients (hospitalized from the emergency room or outpatient clinics) and 39 (45%) as outpatients. There were no significant associations between gender and test results (p=0.205). The mean age of the patients

Table 1. Distribution of outpatients and inpatients by age

Age range (y)	Inpatients (%)	Outpatients (%)	No. of patients
0-1	6 (12.8%)	5 (12.8%)	11 (12.8%)
2-5	19 (40.4%)	6 (15.4%)	25 (29%)
6-10	8 (17%)	4 (10.3%)	12 (14%)
11-16	0	2 (5.1%)	2 (2.3%)
>16	14 (29.8%)	22 (56.4%)	36 (41.9%)
Total	47 (100%)	39 (100%)	86 (100%)

Table 2. Distribution of single causative agents

Single agent	n	%
Viral		
Adenovirus	3	14.3%
Astrovirus	4	19.05%
Norovirus G1	2	9.5%
Norovirus G2	7	33.3%
Rotavirus	4	19.05%
Sapovirus	1	4.8%
Total	21	100%
Bacterial		
verocytotoxin-producing *Escherichia coli (VTEC)*	2	10%
Shigella spp./Enteroinvasive Escherichia coli (EIEC)	3	15%
Salmonella spp.	10	50%
Campylobacter coli/jejuni	3	15%
Clostridium difficile	2	10%
Yersinia enterocolitica	0	0
Total	20	100%

Microscopic examination showed leukocytes in 49 of the 86 samples (57%), and erythrocytes in 20 (23%). In 53.5% of the stool samples subjected to FTD viral gastroenteritis and FTD bacterial gastroenteritis multiplex PCR tests, a causative agent could be detected. Among patients in whom a causative agent could be detected, 72% were under 16 years of age and 28% were over 16 years of age.

was 22.6 years (range: 2 months to 88 years), while the median age was 7.6 years; 41.9% of the patients were older than 16 years of age, and half of those under 16 years of age were aged between 2 and 5 years. Table 1 shows the distribution of inpatients and outpatients according to age groups. The test results were categorized separately for patients ≥ 16 and < 16 years of age.

In the former group of patients, the causative agent was viral in 55% and bacterial in 45%, while the corresponding percentages were 54% and 46% in the latter group of patients. A significant association between age (< 16 vs. ≥ 16 years) and test results was found (p=0.012). A single pathogen was identified in 41 of the 46 samples in which a causative agent was found, and two agents were identified in the remaining 5 samples (11%).

Table 3. Distribution of multiple causative agents

Multiple agents	n
verocytotoxin-producing Escherichia coli (VTEC)+ Salmonella spp.	1
Norovirus G1+ Norovirus G2	3
Rotavirus+ Sapovirus	1
Total	5

Table 4. Frequency and distribution of patients and causative agents

	0-1 y	2-5 y	6-10 y	11-16 y	>16 y	Total
Adenovirus	1	2	-	-	-	3 (6.52%)
Astrovirus	2	-	-	-	2	4 (8.70%)
Norovirus G1 +Norovirus G2	-	1	-	1	1	3 (6.52%)
Norovirus G2	-	3	1	-	3	7 (15.20%)
Norovirus G1	1	1	-	-	-	2 (4.34%)
Rotavirus+ Sapovirus	-	-	1	-	-	1 (2.20%)
Rotavirus	1	-	2	-	1	4 (8.70%)
Sapovirus	1	-	-	-		1 (2.20%)

verocytotoxin-producing Escherichia coli (VTEC)	-	-	-	-	2	2 (4.34%)
Shigella spp. /Enteroinvasive Escherichia coli (EIEC)	-	1	1	-	1	3 (6.52%)
Salmonella spp.	-	5	5	-	-	10 (21.70%)
verocytotoxin-producing Escherichia coli (VTEC)+ Salmonella spp.	-	1	-	-	-	1 (2.20%)
Campylobacter coli/jejuni	-	2	-	-	1	3 (6.52%)
Clostridium difficile	-	-	-	-	2	2 (4.32%)
Yersinia enterocolitica	-	-	-	-	-	-
Total no. of positives	6 (13%)	16 (35%)	10 (22%)	1 (2%)	13 (28%)	46 (100%)
Total no. of patients	11 (12.8%)	25 (29%)	12 (14%)	2 (2.3%)	36 (41.9%)	86 (100%)

Viral causative agents were as follows in the decreasing order of frequency: *Norovirus G2, Rotavirus and Astrovirus, Adenovirus, Norovirus G1, Sapovirus*. Bacterial agents that were identified were as follows in decreasing order: *Salmonella spp., Shigella spp. /EIEC and Campylobacter coli/ jejuni*, verocytotoxin producing *Escherichia coli (VTEC)* and *Clostridium difficile* (Tables 2, 3). Overall, the most frequent causative agents were as follows with decreasing order of frequency: *Salmonella spp.; Norovirus; Rotavirus and Astrovirus; Shigella spp. / EIEC, Adenovirus and Campylobacter coli/ jejuni;* verocytotoxin producing *Escherichia coli (VTEC) and Clostridium difficile.* The distribution of causative agents according to age groups is shown in Table 4.

DISCUSSION

Molecular microbiological diagnostic methods recently introduced into clinical practice are playing an increasingly important role by allowing clinicians to administer more appropriate and effective treatments for their patients thanks to their ability to provide rapid and accurate diagnostic information as compared to more conventional diagnostic approaches (15). Several studies have shown improved rates of detection of the causative agents with the use of molecular methods in patients with gastroenteritis (16-19). In our study, a causative agent could be detected and appropriate treatment could be administered in 38%, 70%, and 53.5% of the patients ≥ 16 years of age, < 16 years of age, and in the overall patient group, respectively, when multiplex PCR method was used for diagnosis. In 2018, a Spanish study by Martin et al., the causative agent could be detected in only 27.7% of their patients with diarrhea when conventional methods were used, as compared to a detection rate of 66.2% with multiplex PCR (20). In another study from Italy by Piralla et al., at least one causative agent could be identified in 54.8% of the overall study population when the film-array method was utilized for diagnosis in patients aged 0 to 80 years with acute gastroenteritis (73.2%) and in patients aged < 18 years with bloody diarrhea (26.8%). In that study, a causative agent could be detected in 47.9% of the patients with acute gastroenteritis and in up to 73.3% of the patients with bloody diarrhea (confirmed by additional methods) (17). Although these figures are close to our observations, several patient characteristics such as inpatient vs. outpatient care or age group should be taken into consideration when comparing the results. In a 2018 study from Taiwan by Huang et al., bacterial, viral and/or parasitic organisms could be identified in 40% of the cases with conventional cultures or PCR, while this figure was 56% when multiplex PCR (Luminex xTAG gastrointestinal panel) was used (18). In our study, 66% of patients that were under 16 years of age were treated as inpatients and a causative organism could be detected in 70% of them. On the other hand, 38% of the patients ≥ 16 years of age were inpatients and a causative agent could be identified in 38%. Of our overall population, 55% were treated as inpatients, among whom a causative organism was identified in 53%, contributing to treatment and management. Similar to our findings, Göktaş et al. also reported an increased detection rate among their inpatients when compared to outpatients (21).

When compared with conventional diagnostic approaches in both adult and pediatric populations, molecular multiplex PCR panels have been reported to provide significant benefits in terms of shortened diagnostic and therapeutic delays as well as decreased duration of hospitalization. Appropriate and as needed use of antibiotics is associated with decreased length of inpatient care and prevention of outbreaks via rapid isolation of the patient, particularly in the pediatric age group. However, since our study was not designed to evaluate the cost-efficacy of this approach, we have not been able to provide detailed information in this respect.

In our study, two causative organisms were identified in only 5 samples (11%), of which 1 was from a patient ≥ 16 years of age and 4 were from patients < 16 years of age. In contrast, in a study by Eibach et al. conducted with the participation of adult patients in Ghana, co-infection was detected in most of the diarrheic stool samples. This finding was explained with the high rates of exposure to

environmental pathogens among asymptomatic children dwelling in conditions of poor hygiene and health, and the authors questioned the value of highly-sensitive multiplex PCR methods for the diagnosis of gastrointestinal infections in sub-Saharan Africa (7). As compared to our study, the reported rates of co-infections were higher in Italy (28.2%),(17) significantly lower in Holland (0.9%), and similar in the US (14.1%) (19, 22). In addition, there was significant resemblance between the above-reported populations and our patient population in terms of hygiene and sanitation, suggesting that PCR-based tests may be considered for routine use in certain geographical locations and patient populations.

Although leukocytes and erythrocytes are more commonly observed in stool microscopy of samples from inflammatory gastroenteritis cases, this examination is not reliable for the differentiation between inflammatory and non-inflammatory gastroenteritis (23). In our study, 57% of the stool samples (49/86) had leukocytes, and 23% (20/86) had erythrocytes, similar to general literature data (14, 17).

In the current study, the overall frequency of viral and bacterial organisms was almost equal. The most frequently isolated viral and bacterial organisms were Norovirus, and Salmonella spp., respectively. In the study by Huang et al., an organism could be identified in 55% of the samples, of which 67.7%, 31.4%, and 3.3% were reported to be bacterial, viral, and parasitic in origin. While the results of these authors are comparable to ours with respect to the fact that most of the causative organisms were bacterial or viral, they differed from ours, since bacterial agents were more common in that study. In the developing world, most cases of gastroenteritis are due to bacteria, followed by parasites, and viruses. In our study, viral agents were identified at a high frequency (50%), comparable to the reports from developed countries. In a study by Göktaş et al. conducted in Istanbul, the most frequently isolated organisms were of bacterial origin (31.6%), while viruses were responsible from only a small fraction of the patients (3.2%) (21). Again in another study from Istanbul, Keşke et al. identified high rates of bacterial organisms in both adults (83%) and pediatric patients (74%) using a rapid molecular test panel (Biofire's FilmArray System) (14). The variance between the studies from the same province was accounted for by the difference in patient populations and/or utilization of different molecular panel tests. In a pediatric study by Onori et al. from Italy, viral and bacterial organisms were responsible for 68% and 32% of the cases, respectively (24).

In a Taiwanese study involving all age groups and utilizing multiplex PCR (Luminex xTAG) methodology, the following organisms were isolated in decreasing frequency: *Salmonella spp., Norovirus G1/G2, Clostridium difficile toxin A/B, and Campylobacter* (18). Although bacterial agents were more frequent in that study (67.7%) than ours, it is interesting to note that the order of frequency was the same with our observations, as long as the most frequently isolated bacterial and viral agents are considered. In a study from Italy by Piralla et al. in which bacterial agents were isolated in 50% of the samples, *Rotavirus, Campylobacter, C.difficile, Norovirus, Salmonella, and enteropathogenic E.coli (EPEC)* were the most frequently isolated organisms, at odds with our findings (17). Again, in a multi-center study from Europe by Spina et al. involving a group of subjects treated on an outpatient basis, at least one causative agent was found in 54.2% of the patients, of which 83% were bacterial in origin. Again, contrary to our findings, *EPEC, Campylobacter, toxigenic C.difficile, enteroaggregative E.coli (EAEC), Norovirus and enterotoxic E.coli (ETEC)* species were the most commonly isolated organisms (25). There was more close resemblance between our findings and those of the Taiwanese study in terms of the type of causative organisms, despite Italy's closer geographical location to Turkey as a Mediterranean country, suggesting that different organisms may well be responsible for these infections even in geographically closer areas. Previous studies using molecular methods in Turkey mostly focused on the incidence of viral organisms (4, 9, 26). Furthermore, other authors investigated other types of causative agents (3, 27). One of the scarce studies from our country by Keşke et al. also reported different results from our study by showing a coinfection rate of 42.4% in adults and 36.8% in pediatric patients among an overall group of subjects, 36% of whom were treated as inpatients and 71% of whom had at least one causative agent (14). In that study utilizing the FilmArray™ methodology, the most frequent organisms in pediatric patients were *EPEC, C.difficile, Norovirus, EAEC, Shiga*-like toxin producing *E.coli (STEC), ETEC, Campylobacter, Salmonella,* and *Rotavirus;* and the most commonly isolated organisms in adults were *EPEC, EAEC, ETEC, STEC, Norovirus, Campylobacter spp, Salmonella spp, and C.difficile* in decreasing order. That study differs from our study in that both groups had a high frequency of E.coli species and relatively low frequencies of Salmonella species and viral agents. In the study by Göktas et al. using multiplex PCR method, isolated causative agents in single-agent infections were as follows in decreasing order: *Salmonella spp., Gardia lamblia, EHEC, Norovirus, Campylobacter spp/C. difficile toxin B* (21). That study differed from ours by both detecting a higher rate of co-infections, i.e. 22%, and also by showing a different distribution of causative agents. Despite inclusion of similarly aged subjects from the same location (i.e. Istanbul) such differences were noted, which may be related with the higher number of patients with acute gastroenteritis who required hospitalization or with the detection of different agents by different molecular systems. Since the Fast-track panel did not include the parasitic agents at the time of study conduct, we have not been able to report relevant data. Further data may be collected with the introduction of multiplex PCR tests that also allow identification of parasitic organisms.

Multiplex molecular diagnostic tests play an important role in epidemiological surveillance, investigational projects, and in assessing disease outbreaks; however, such data were not presented due to the scope of our study. However, as our results suggest, better characterization of complicated cases requiring hospitalization may allow administration of efficacious treatments, while avoiding inappropriate antibiotherapy and its side effects (7). There may be a particular need for the use of molecular diagnostic methods in the elderly, young children, or immunocompromised subjects presenting with fever, bloody diarrhea, and dehydration, for rapid and accurate detection of the causative organisms (18).

Our observations and previous experience suggest that multiplex panel tests represent a potential breakthrough in microbiology that may lead to a complete transformation in our conventional techniques and approaches. When the pros and cons of these novel tests are considered, we should first mention their current status as a routine clinical test. In our institution where these methods are in the reimbursement list, we have been able to receive positive feedback from clinicians with regard to clinical utility and performance of this methodology. Although they are more expensive than conventional methods, the cost issue may be perceived as a rather secondary element, as long as the needs of the clinicians are met and their problems are solved. Such benefits actually represent the "indirect advantages", which may ultimately lead to their cost-effective use.

Gastrointestinal infections pose certain difficulties such as the low microbiological success rate, delayed diagnosis, and challenges in the identification of the causative organism(s). In many instances, laboratories exclude certain tests from their services due to cost issues. However, the results of the current bacteriological culture tests cannot be obtained earlier than 2 or 3 days. Furthermore, laboratories do not have much to offer in case of gastroenteritis of viral origin. Most of the available antigenic tests have low sensitivity and specificity. Panel tests hold the potential to address the entirety of such problems, allowing rapid, simple, and accurate identification of multiple organisms, and hence appropriate treatment. These tests prevent inappropriate use of antibiotics, pave the way for infection control measures, and provide accurate epidemiological data, shedding light into a dark area.

Although test panels incorporating most potential gastrointestinal pathogens have been introduced, currently some causative agents could not be integrated into certain panel tests (*eg. Aeromonas spp., Dientamoeba fragilis etc*.). However, it is now clear that these panel tests will be/are becoming the diagnostic test of choice in patients with diarrhea, who would require separate conventional laboratory procedures for a variety of protozoa, viruses, and bacteria.

CONCLUSION

In addition, these tests will provide important local epidemiological data to outline empiric antibiotic use in antibiotic management guidelines. However, assessment of the antibiotic susceptibility remains an important component, particularly of epidemiological data collection. In such cases, a kind of "reflex culture testing" may be considered. Appropriate and rational use of these tests, and provision of adequate consultation and/or reporting to clinicians with the interpretation of the test results should be among the targets of clinical microbiologists. It appears that, the priorities after determining the incidence of microorganisms should include development of algorithms allowing routine use of these relatively expensive tests, as well as multiple-stage or masking approaches to categorize organisms according to their potential role in these infections. This warrants further studies. It may be predicted that their cost will decrease over time with more widespread use.

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Melda Ozdamar ORCID: 0000000335329255
Berrin Uzun ORCID: 0000000191155910
Salih Turkoglu ORCID: 0000000197610451

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