Original Research Paper

Epidemiology and Detection of Bacterial Diarrhea Using Multiplex Polymerase Chain Reaction, Focusing on *Campylobacter* spp.

[1]Eun Ju Oh, [2]Tae Su Jang, [3]Jung Min Park and [4]Jae Kyung Kim

[1]*Department of Medical Laser, Dankook University Graduate School of Medicine, Republic of Korea*
[2]*Department of Health Administration, Dankook University College of Health and Welfare, Republic of Korea*
[3]*Department of Public Health Science, Dankook University Graduate School of Natural Science, Republic of Korea*
[4]*Department of Biomedical Laboratory Science, Dankook University College of Health Sciences, Korea*

Abstract: This study assessed the clinical characteristics, epidemiological trends, and changes associated with major pathogens causing gastrointestinal inflammation. We retrospectively analyzed the test results of hospitalized patients who were administered a mPCR test for diarrhea causing bacteria between June 2010 and December 2019 at the Cheonan Dankook University hospital in the Republic of Korea and identified clinical characteristics and epidemiological trends related to major pathogens causing gastrointestinal inflammation. Our results showed that infections due to *Clostridium perfringens*, *Escherichia coli* H$_7$, *Campylobacter* species (spp.), and *Clostridioides difficile* toxin B varied seasonally (March to October). When examining annual trends, we found that *Escherichia coli* H$_7$ incidence decreased significantly throughout the time frame examined, from 27.27% of total infections in 2010 to 8.93% in 2019. In contrast, the incidence of both *Clostridium* spp., and *Campylobacter* spp. gradually increased throughout this time frame. Our study was able to demonstrate that improvements in environmental hygiene have aided in reducing the occurrence of water borne diarrhea, while infections due to food borne, anaerobic and diarrhea causing bacteria, such as those present in non-sterile milk and poultry, are increasing. We believe that this study will serve as a useful resource for better understanding the dynamics and risks associated with bacterial diarrhea, including infections caused by *Campylobacter* spp.

Keywords: Acute Diarrhea, *Campylobacter*, Clostridoides Difficile, *Clostridium perfringens*, *Escherichia coli* H$_7$

Introduction

Acute diarrhea is reported in approximately 3-5 billion people annually, with an annual global mortality of approximately 2 million (Kim *et al*., 2013; World Health Organization, 2015). The World Health Organization (WHO) estimated that there are approximately 2 billion diarrheal episodes per year and approximately 1.9 million deaths of children under the age of five each year in 2013, accounting for approximately 525,000 deaths in 2017 and 370,000 deaths in 2019 among children under the age of five worldwide (World Health Organization, 2017). Acute diarrhea caused by bacteria and viruses is a water and food borne disease transmitted through the fecal oral route. Contaminated food and water have the potential to infect a large number of people simultaneously, causing symptoms such as vomiting, diarrhea, and abdominal pain. Infection may also lead to socioeconomic damage owing to patient treatment and hospitalization (Seo and Choi, 2010; Shim, 2012). In developed nations, such as Western Europe, the United States, and Japan, the common bacterial pathogens causing food borne diarrhea are *Campylobacter* spp. and *Salmonella* spp., with the highest prevalence of diseases reported in children under five years of age (Barrett and Fhogartaigh, 2017). Diarrhea in children is a serious health hazard in developing countries caused by various microorganisms (Sayyahfar *et al*., 2022). *Campylobacter* spp. are a major cause of bacterial infection, with approximately 800,000

cases annually in the United States alone (Scallan *et al*., 2011; 2015). *Campylobacter* spp. transmission occurs mainly through poultry and contaminated water (Humphrey *et al*., 2007; Taylor *et al*., 2013). Our investigation conducted in the Republic of Korea found that *Salmonella* spp., dysentery, and *Escherichia coli* were the main causes of bacterial enteritis up until the early 2000s (Pai, 2007; Kim *et al*., 2013). However, social and environmental factors and trends, such as increased consumption of food away from home (e.g., school meals during childhood and adolescence and dining out), overseas travel, changes in living conditions, and climate change, have promoted changes in bacterial pathogen dynamics (Van Lint *et al*., 2015). Recently, an increase in the incidence of *Campylobacter* spp. infections have been reported, but these studies were limited and tailored to Europe in terms of population and infection trends (Kuhn *et al*., 2018); moreover, few current reports have examined bacterial infections within the Korean population (Lake *et al*., 2019). The disparity between the characterization of disease in developed countries versus in Korea is likely due to difficulties associated with the isolation and identification of *Campylobacter* spp., which makes it difficult to diagnose diseases associated with it. However, recent developments in molecular biology techniques have improved our ability to characterize bacterial pathogens (Ricke *et al*., 2019). In large medical institutions, testing techniques, such as nested PCR, real-time quantitative PCR, digital PCR (dPCR), and Next Generation Sequencing (NGS), are widely used for diagnosis. In this study, the separation frequency of *Clostridioides difficile* and *Campylobacter* spp., which are anaerobic bacteria that cause diarrhea, was analyzed from fecal samples obtained between June 2010 and December 2019, using multiplex Polymerase Chain Reaction (mPCR). Our findings would enhance the treatment and prevention of gastroenteritis with diarrhea by providing knowledge of the epidemiology and clinical characteristics of *Campylobacter* spp. and *Clostridioides difficile* toxin B infections.

Materials and Methods

Sample Collection

This study retrospectively analyzed the test results of patients who were hospitalized and administered a mPCR test for diarrhea causing bacteria from June 2010 to December 2019 at the Cheonan Dankook University hospital in the Republic of Korea. A total of 2,300 fecal samples were tested for bacterial species that cause diarrhea using mPCR. Nucleic acids were extracted from the samples within 24 h. Data on the age and sex of the study participants were retrieved from patient records. We were unable to collect information regarding the incubation period of the bacteria due to the retrospective nature of the study and because the time of the hospital visit after disease onset varied among patients.

Ethical Approval

This study was conducted in accordance with the principles of the declaration of Helsinki and was approved by the Institutional review board of Dankook University, Republic of Korea (approval number: 2020-01-012). Owing to the retrospective nature of the study, the Institutional review board waived the requirement for informed consent.

Nucleic Acid Extraction

A specimen of 200 μL was used for DNA isolation; DNA isolation for mPCR was performed using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) in accordance with the manufacturer's instructions.

PCR Analysis

Seeplex Diarrhea B1 and B2 ACE detection kits (Seegene, Korea) were used for mPCR. Eleven pathogens, including *Aeromonas* spp., *Clostridium perfringens*, *E. coli* H$_7$, *Campylobacter* spp., *Clostridioides difficile* toxin B, *E. coli* O157, *Shigella* spp., *Salmonella* spp., verocytotoxin producing *Yersinia enterocolitica*, *Vibrio* spp., and *E. coli*, were evaluated. Of the 11 pathogens considered, the following four were selected and analyzed: *Clostridium perfringens, E. coli* H$_7$, *Campylobacter* spp., and *Clostridioides difficile* toxin B. As the detection of *Yersinia enterocolitica* and *Vibrio* spp. was extremely rare, these bacterial species were excluded from our analysis, which focused mainly on anaerobic bacteria. The PCR product sizes and target genes are listed in Table 1.

Electrophoresis was performed on a 2% agarose gel containing ethidium bromide at 100 V for 30 min. The results were analyzed after photographs were acquired.

Statistical Analysis

SAS v. 9.4 (SAS institute Inc., cary, NC, USA) was used to perform statistical analyses. Statistical significance was set at $p < 0.05$.

Table 1: Product sizes and their target genes from mPCR

Pathogen	Product size (bp)	Target genes
Clostridium perfringens	700	Cpa
Clostridioides difficile toxin B	476	tcdB
E. coli O157: H$_7$	370	fliC
Campylobacter spp.	227	asp, hip

Note: mPCR, multiplex polymerase chain reaction; spp., species; E. coli* O157: H$_7$, *Escherichia coli* O157: H$_7$

Results

Of the 2,300 patients considered, 1, 265 (55.0%) were male and the positive rate of bacterial diarrhea infection was 1,030 (44.8%). The positive rate of *Clostridioides difficile* toxin B was 6.0% in males (76/1, 265) and 7.9% in females (82/1, 035). The positive rate of *Clostridium perfringens* was 7.35% in males (n = 93) and 6.7% (n = 69) in females. *Campylobacter* spp. positive rate was 7.8% (n = 98) in males and 7.2% (n = 74) in females, while that of *E. coli* H_7 was 13.7% (n = 173) in males and 12.38% (n = 128) in females (Table 2). The average positive rate of *Clostridium perfringens, E. coli* H_7*, Campylobacter* spp., and *Clostridioides difficile* toxin B in males was 55.5% (440/793), whereas, in females, it was 44.5% (353/793).

Of those aged <10 years, 33.0% (n = 262) of patients were positive for *Clostridium perfringens*, *E. coli* H_7, *Campylobacter* spp., or *Clostridioides difficile* toxin B. We observed a higher positive rate of *Clostridium perfringens* and *Clostridioides difficile* toxin B in the <10 years age group (n = 107, 33.4%) than in the >70 years age group (31.0%, 99/320), Table 2. The positive rates of *Clostridioides difficile* toxin B and *Clostridium perfringens* were not significantly associated with age (P = 0.279) (Table 2). We observed a higher positive rate of *Campylobacter* spp. in the 10-19 years age group (34.3%, 59/172) than in the <10 years age group (27.3%,

47/172), Table 2. *Campylobacter* spp. positive rate was significantly associated with age (P = 0.002*) (Table 2). We observed a higher positive rate of *E. coli* H_7 in the <10 years age group (35.9%; 108/301) than in the >70 years age group (17.9%, 54/301), Table 2. The positive rate of *E. coli* H_7 was significantly associated with age (P = 0.034*) (Table 2).

The overall positive rate of *Clostridium perfringens* and *Clostridioides difficile* toxin B peaked in June at 11.9% (38/320), whereas the lowest rate (5.3%) occurred in January and February (17/320). The overall positive rate of *Campylobacter* spp. was highest in June (20.9%, 36/172) and lowest in January (1.2%, 2/172). The overall positive rate of *E. coli* H_7 was highest in June (13.3%, 40/301) and lowest in March (3.7%, 11/301) (Fig. 1).

An assessment of the annual distribution of *Clostridium perfringens, E. coli* H_7*, Campylobacter* spp., and *Clostridioides difficile toxin* B infections between June 2010 and December 2019 showed that the overall positive rates of *Clostridium perfringens* and *Clostridioides difficile* toxin B were highest in 2017 (26.32%, 55/209) and lowest in 2010 (6.49%, 5/77) (Fig. 2). The overall positive rate of *Campylobacter* spp. was highest in 2018 (11.36%, 31/273) and lowest in 2013 (3.47%, 14/404) (Table 2). The overall positive rate of *E. coli* H_7 was highest in 2010 (27.27%, 21/77) and lowest in 2018 (7.33%, 20/273) (Fig. 2).
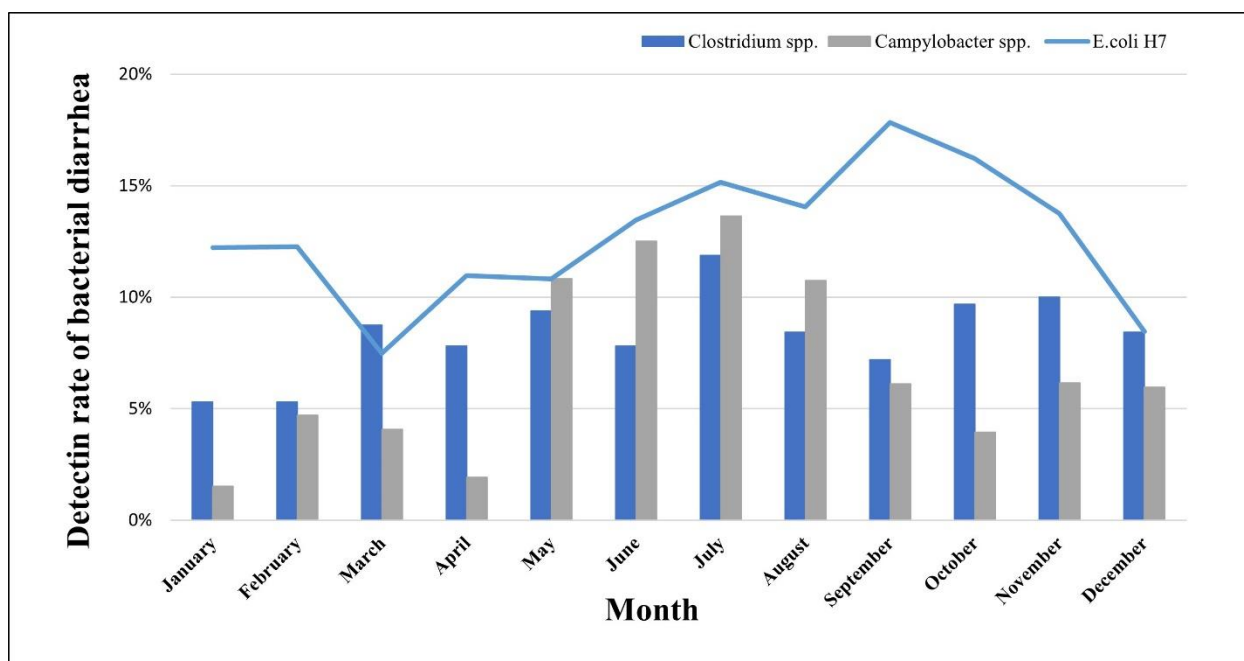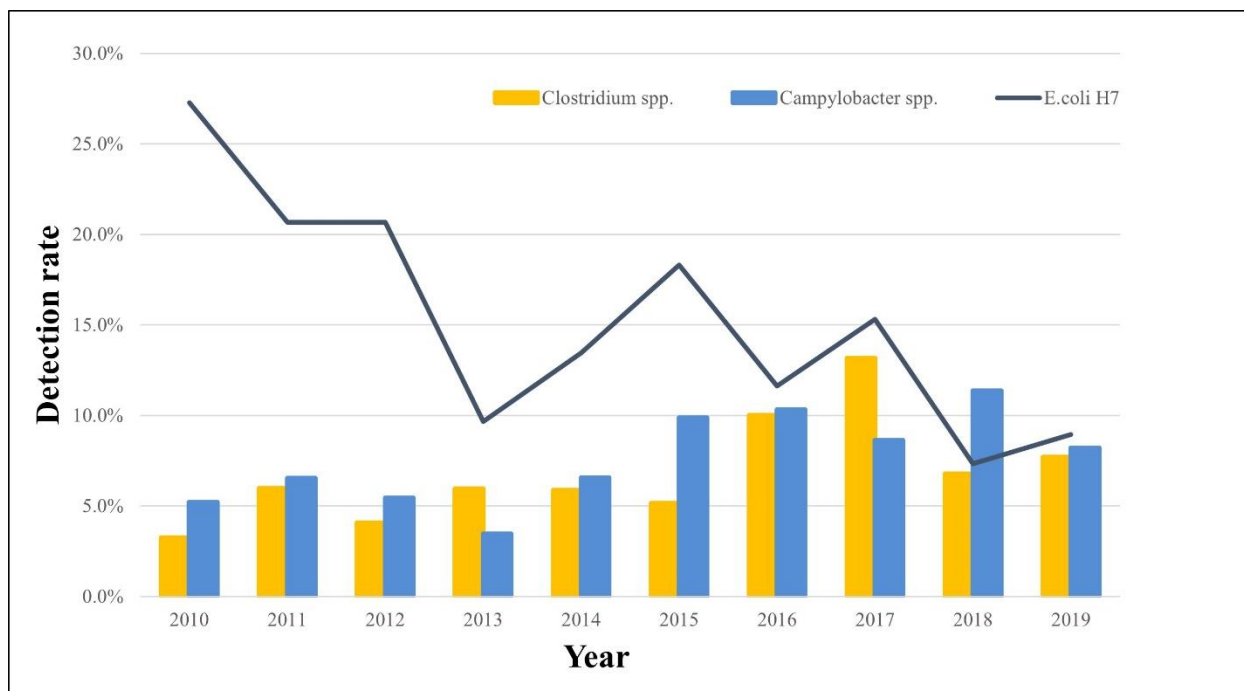

Fig. 1: Monthly detection rates of bacterial diarrheal infections (2010-2019)

Fig. 2: Detection rates of bacterial diarrheal infections (2010-2019)

Table 2: Age and sex distribution of bacterial diarrhea positive rates and correlation between age and bacterial diarrhea positive rates between 2018 and 2020

	Infected with *Campylobacter* spp.		Infected with *Clostridium* spp.		Infected with *E. coli* H_7	
Sex	N	%	N	%	N	%
Male	98	57.0	169	52.8	173	57.5
Female	74	43.0	151	47.2	128	42.5
Total	172	100.0	320	100.0	301	100.0
Age	No.	%	No.	%	No.	%
<10	47	27.3000	107.000	33.4	108	35.9
10-19	59	34.3000	20.000	6.2	52	17.3
20-29	35	20.3000	10.000	3.1	12	4.0
30-39	4	2.3000	10.000	3.1	13	4.3
40-49	9	5.2000	12.000	3.8	22	7.3
50-59	4	2.3000	26.000	8.1	20	6.6
60-69	6	3.5000	36.000	11.3	20	6.6
≥70	8	4.7000	99.000	31.0	54	17.9
Total	172	100.0000	320.000	100.0	301	100.0
p-value	0.0017[**]	0.2788	0.034[*]			

[*]$p < 0.05$; [**]$p < 0.01$

*Note: spp., species; *E. coli* H_7, *Escherichia coli* H_7

Discussion

In the past, a common way to diagnose bacterial infections in diarrhea was to separate culture pathogens (Fan *et al.*, 2008; Khamrin *et al.*, 2011). However, recent molecular biological techniques have facilitated quick and accurate detection of the anaerobic diarrhea causing bacteria that are difficult to culture. Although reports of the prevalence of anaerobic diarrhea causing bacteria are increasing in Korea, few long-term studies have reported on the epidemiology of diarrhea causing bacteria identified at a single institution. This study analyzed pathogens in the fecal samples of patients with gastroenteritis, which were collected from June 2010 to December 2019, and analyzed them using mPCR. We assessed the clinical characteristics, epidemiological trends, and changes associated with major pathogens causing gastrointestinal inflammation.

A previous study (Kim et al., 2013) reported that the incidence of bacterial infections in Korea is highest between June and September, but the prevalence of *Clostridium perfringens, E. coli* H$_7$, *Campylobacter* spp., and *Clostridioides difficile toxin* B differed between March and October. This observation is in agreement with that of a previous report (Korea Disease Control and Prevention Agency, 2016), which stated that acute diarrhea tends to occur throughout the year due to a combination of factors such as climate change, traffic and development, overseas travel, and restaurant growth. These factors reduce seasonal variation in the prevalence of bacterial disease; additionally, variation in infection prevalence is also reduced due to the maintenance of constant indoor temperatures throughout the year.

As indoor living conditions improve, the use of both cooling and heating systems, such as boilers and air conditioners, is expected to increase. The incidence of diarrhea is especially high in infants, children, adolescents, and older adults, who spend most of their time indoors (Korea Disease Control and Prevention Agency, 2016; Goh et al., 2019). In this study, *Clostridium perfringens*, *E. coli* H$_7$, and *Clostridioides difficile* toxin B were the most prevalent in children and older adults. *Campylobacter* spp. were the most prevalent among young people aged >10 ~ <30 years. A possible explanation for the observed high incidence of *Campylobacter* spp. infections in teens and individuals in their twenties versus children or older adults, who are known to have weak immune systems, is that these age groups consume more poultry (Allos, 2001; Schielke et al., 2014).

During the study period, the positive rate of *E. coli* H$_7$ decreased significantly to 8.93% in 2019 from its peak of 27.27% in 2010, while the positive rate of *Clostridium* spp. and *Campylobacter* spp. increased. This is similar to the trends observed in other studies (Allos, 2001; Geissler et al., 2017; Colomb-Cotinat et al., 2019), which have reported an increase in the abundance of anaerobic bacteria. This suggests that infections caused by anaerobic diarrhea causing bacteria through food sources, such as consumption of non-sterile milk and poultry, are increasing, while water borne diarrheal diseases are decreasing due to improvements in environmental hygiene (Allos, 2001; Geissler et al., 2017).

The world health organization has defined *Campylobacter* spp. as the most common cause of gastroenteritis with diarrhea. Antibiotic treatment is not required for general bacterial enteritis but may be required for infants due to intestinal complications (Kim and Chung, 2020). Therefore, age, clinical symptoms, and inflammatory indicators should be considered and special care and continuous epidemiological monitoring are required as per medical and public health guidelines. In the United States, data on bacterial diarrhea, especially diseases caused by *Campylobacter* spp., are regularly reported to the food borne diseases active surveillance network by surveillance sites (Centers for Disease Control and Prevention, 2014). In Canada, quality specific surveillance data are collected through C internet (Kim and Chung, 2020). National management is also performed in Australia, but no common guidelines, standardized inspection procedures, monitoring methods, or reporting guidelines are used. We propose the use of international monitoring and standardization procedures for bacterial diarrhea to improve preventive and control measures of geographical predictors and risk factors associated with bacterial diarrhea.

This study had some limitations. First, it was performed within a single institution and did not constitute all areas of Korea. In addition to the pathogenic nature of the viral infection, pathogenic epidemics are closely related to geographical and climatic variations, as well as socioeconomic factors (Liu et al. 2015; 2019). Therefore, disparities in the geographical location of patients could have led to differences in the detection rates of *C. perfringens*, *E. coli* H$_7$, *Campylobacter* spp., and *Clostridioides difficile* toxin B observed in this study.

Second, some of the samples may have been provided by patients who were not residents in the study area because sample data were anonymously processed. Third, this was a retrospective rather than a prospective study and the results were not analyzed while considering the clinical characteristics of patients. Thus, future studies that take into account the clinical characteristics of patients are required. Fourth, only bacterial diarrhea infection trends were analyzed and further research will be needed to evaluate the effects of other pathogens that cause diarrhea. Finally, since the patients included in this study had acute gastroenteritis, those with mild symptoms or those who were asymptomatic were not investigated. It is necessary to thoroughly investigate the regional and temporal shifts affecting the prevalence of diarrhea causing bacteria based on more objective indicators.

Despite these limitations, our study identified differences in the positive rates of bacterial diarrhea on season and patient age. Characterization of these differences will be useful for explaining the dynamics and risk factors for bacterial pathogens that cause diarrhea, including *Campylobacter* spp., particularly for improving disease prevention and control measures. In the future, extensive research using more data and molecular biology and epidemiological studies is warranted. It is expected that more objective indicators of change in the prevalence of diarrhea causing bacteria and continuous studies of bacterial infection prevention and management measures will be needed.

Conclusion

This study presented practical evidence for understanding the differences in the positive rates of bacterial diarrhea based on the season and age of patients. Our findings demonstrated that improvements in environmental hygiene have helped reduce the occurrence of water borne diarrhea, while infections due to food borne, anaerobic and diarrhea causing bacteria, such as those present in non-sterile milk and poultry, are increasing. We believe that this study provides useful data for a better understanding of the epidemiology and risks associated with bacterial diarrhea, including *Campylobacter* spp. infections.

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Funding Information

Author's Contributions

Eun Ju Oh and Jae Kyung Kim: Made substantial contributions to the conception and designed of the study. These authors contributed equally to this study.

Tae Su Jang and Jung Min Park: Made substantial contributions to the acquisition and analysis of the data.

Ethics

This study was approved by the Dankook University Institutional Review Board (IRB No. 2020-01-012).

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