

Comparison of the Detection Limits of the Culture and PCR Methods for the Detection of *Clostridium difficile*, *Clostridium perfringens*, *Campylobacter jejuni*, and *Yersinia enterocolitica* in Human Stool

Leila Ganji,¹ Masoumeh Azimirad,² Nastaran Farzi,^{2,3} Masoud Alebouyeh,^{2,3,*} Mohammad Hassan Shirazi,^{1,*} Seyed Saeed Eshraghi,¹ Abbas Mirshafiey,¹ Naser Ebrahimi Daryani,¹ and Mohammad Reza

Zali^{2,3}

¹Department of Pathobiology, School of Public Health, University of Medical Sciences, Tehran, Iran

²Foodborne and Waterborne Diseases Research Center, Research Institute for Gastroenterology and Liver Diseases, Shahid Beheshti University of Medical Sciences, Tehran, Iran

³Gastroenterology and Liver Diseases Research Center, Research Institute for Gastroenterology and Liver Diseases, Shahid Beheshti University of Medical Sciences, Tehran, Iran

*Corresponding authors: Masoud Alebouyeh, Foodborne and Waterborne Diseases Research Center, Research Institute for Gastroenterology and Liver Diseases, Shahid Beheshti University of Medical Sciences, Tehran, Iran, E-mail: masoudaleboyeh@gmail.com.; Mohammad Hassan Shirazi, Department of Pathobiology, School of Public Health, University of Medical Sciences, Tehran, Iran, E-mail: mhshirazi@tums.ac.ir

Received 2016 May 01; Revised 2016 July 20; Accepted 2016 August 02.

Abstract

Background: Detection of fastidious enteropathogenic bacteria in fecal samples of patients with gastroenteritis is a challenge in clinical microbiological laboratories.

Objectives: The aim of this study was to compare the detection limits of the PCR and culture methods for the diagnosis of *Campylobacter* spp., *Yersinia* spp., *Clostridium perfringens*, and *Clostridium difficile* in human stool samples.

Methods: Healthy human stool and sterile phosphate-buffered saline (PBS) samples were separately spiked with 10-fold dilutions of *C. jejuni*, *C. difficile*, *Y. enterocolitica*, and *C. perfringens* reference strains to obtain final concentrations of 10^1 - 10^8 colony forming units (CFU) per gram. Dilutions of each suspension were inoculated onto specific culture media and colony counts were determined. Polymerase chain reaction (PCR) was carried out on DNA extracts of each dilution using specific primers. All of the assays were performed in two separate replicas.

Results: In the cases of the culture and PCR assays, detection limits of 10^1 and 102 CFU/g for *C. difficile*, 2×10^4 and 2×10^4 CFU/g for *C. perfringens*, 10^4 and 10^2 CFU/g for *C. jejuni*, and 10^2 and 10^4 CFU/g for *Y. enterocolitica*, respectively, were obtained. In the cases of the spiked PBS samples, a detection limit of 10^1 for *C. jejuni* and *Y. enterocolitica* was obtained using the culture method. While 10^2 -fold higher sensitivity was observed for *C. jejuni* via PCR compared with the culture assay, equal (*C. perfringens*) or lower sensitivity limits (*C. difficile* and *Y. enterocolitica*) were detected for the spiked stool samples with other bacteria.

Conclusions: These results showed differences in the bacterial culture and PCR methods for quantitative detection of fastidious bacteria in human stool samples. However, a bacterial load of 10^4 CFU per gram of stool was measured as a sufficient amount for detection of the fastidious bacteria by either culture or PCR assays. More suitable PCR methods could be used for rapid diagnosis of the slow-growing bacteria in the patients' stool samples.

Keywords: Culture, PCR, *Campylobacter jejuni*, *Clostridium difficile*, *Clostridium perfringens*, *Yersinia enterocolitica*

1. Background

There are increasing data regarding the roles of the intestinal microbiota in the pathogenesis of several human diseases (1). Prolonged interaction of these bacteria with the intestine or their overgrowth seems to be responsible for chronic diseases in this organ (2). Even in low numbers, pathogenic bacteria which are not common members of the gut microbiota can cause different human illnesses under some conditions (3, 4). Invasion of these pathogens into the intestinal barrier layer or the produc-

tion of metabolites that dysregulate the normal signaling pathways of the intestinal cells is involved in the disease progression in infected patients. More than 200 transmitted microbial agents from foodstuffs are associated with gastroenteritis in the human population (5). Among these agents, the most important enteric bacterial pathogens that are responsible for gastrointestinal diseases are *Escherichia coli*, *Campylobacter jejuni*, *Shigella* spp., *Salmonella* spp., *Vibrio cholera*, and *Yersinia* spp. (6). *Clostridium difficile* is also considered the most frequently identified enteric

pathogen in hospitalized patients with a recent history of medication (7). The infectious dose of these pathogens varies depending on their virulence potency and level of resistance to the harsh conditions of the gut environment (8). In some cases, gastrointestinal infectious diseases are acquired by the consumption of contaminated foods or water that are infected with fewer than 10 microorganisms (9). Detection of responsible bacterial agents in environmental or fecal samples mainly depends on the validity of the laboratory tests used and the sampling procedure, including time of sampling and transport conditions. It is hard to resolve challenges that exist for the detection and enumeration of these bacteria, since the causative bacteria are often present in low numbers within these samples or their presence is influenced by food materials or high counts of indigenous bacteria (10). Therefore, a rapid yet sensitive and specific diagnostic assay would be advantageous to clinicians for the early recognition of disease and to infection control practitioners for the swift implementation of control measures (11).

Bacterial culture is considered the “gold standard” for identification of diarrheagenic bacteria from stool specimens. However, this method is time consuming and laborious, requiring prolonged incubation, selective enrichment, and reduction of the background flora, which should be followed by biochemical identification tests (12, 13). Most laboratories are unable to diagnose the anaerobic, microaerophilic, and fastidious bacteria responsible for human gastrointestinal disorders using conventional microbiological methods. Recently, more rapid DNA-based methods for direct identification and even subtyping of these pathogens in stool specimens have been developed (14-21). Polymerase chain reaction (PCR) is a powerful technique for the detection of the target DNA in various clinical specimens, including fecal samples. However, fecal specimens often contain substances that may interfere with the PCR assay, leading to false-positive or false-negative results (2, 4, 11, 13, 15, 22). Accordingly, its application in clinical laboratories needs validation. Comparison of results for conventional culture- and molecular-based methods, which are designed for each bacterial species, is essential.

2. Objectives

In this study, we aimed to compare the detection limit and performance of PCR and culture methods for diagnosis of *Campylobacter* spp., *Yersinia* spp., *C. perfringens*, and *C. difficile*. As enteric pathogens, these are among most sensitive to the ambient culture conditions commonly used for diagnosis in clinical laboratories.

3. Methods

3.1. Bacterial Strains and Growth Conditions

The strains used in this study were the reference strains of *Campylobacter jejuni* (ATCC strain 33560), *C. difficile* (research center of gastroenterology and liver disease [RIGLD]-141), *Yersinia enterocolitica* (ATCC strain 101776), and *C. perfringens* (; RIGLD-2). Selective culture media, including *Brucella* agar (Merck, Germany; supplemented with 5% sheep blood and *Campylobacter* selective supplement), *Clostridium difficile* agar (Mast, UK; supplemented with defibrinated horse blood and *Clostridium difficile* selectavial), *Yersinia* selective agar (Merck; supplemented with *Yersinia* selective supplement CIN), and Egg Yolk agar (Merck; supplemented with neomycin), were used for the subculture of these strains. In the cases of *C. perfringens* and *C. difficile*, the cultures were incubated under anaerobic conditions (Anoxomat, MART Microbiology B.V.; 0% O₂, 10% H₂, 10% CO₂, and 80% N₂) at 37°C for 48 hours. *Campylobacter jejuni* was grown under microaerobic conditions (6% O₂, 6% CO₂, 3% H₂, and 85% N₂) for 24 hours at 42°C; *Y. enterocolitica* was grown under ambient air conditions at 25°C.

3.2. Spiked Stool Experiments

Serial tenfold dilutions of each bacterial species were freshly prepared at defined concentration (10¹ - 10⁸ CFU/mL) in a control stool specimen and phosphate-buffered saline (PBS). A total of 100 µL of each dilution was spread onto the selective media for enumeration of the colony counts. In the cases of *C. difficile*, the inoculated samples were initially treated with alcohol and yeast extract broth to remove the common intestinal microbial flora. For alcohol treatment, about 1 g of stool was mixed with an equal volume of 95% methanol and then slowly vortexed and held at room temperature for 2 minutes. The treated suspensions were cultured on the selective media supplemented with 5% horse blood (CDSA). For the enrichment of *Clostridium*, nearly 1 g of the stool samples were mixed with an equal volume of yeast extract broth (Yeast extract; Merck). The treated suspensions were then cultured on the selective medium. To detect *C. perfringens* spores, the methanol (95%) and heat-treated spiked samples (90°C, 20 minutes) were cultured on Neomycin Egg Yolk agar. Cold enrichment in PBS and direct culture of the inoculated stool samples on CIN (agar 25°C) and MacConkey agar (37°C) were used for isolation of *Y. enterocolitica*. Two replicas of the tests were performed for analysis of the variations in our results (23).

3.3. DNA Extraction and PCR

DNA was extracted from the prepared stools according to the manufacturer's instructions using the DNA Stool

Kit (Bioneer, South Korea). The concentration of DNA samples was 30 µg. All of the DNA extracts were stored at -20°C until use. Amplification of target genes for the detection of *Campylobacter* spp. (16S rRNA), *Yersinia enterocolitica* (ompF), *C. difficile* (Cdd-3), and *C. perfringens* (16S rRNA) was performed using the specific primers depicted in Table 1. All PCR amplifications were performed in 25-µL volumes containing 3 µL of DNA template, 0.5-mM concentrations of deoxynucleoside triphosphates, 2.5 µL of 10 × PCR buffer (gene fanavar), 0.75 mM MgCl₂, 0.3 µM concentrations of each forward and reverse primer, 0.2 U of Taq DNA polymerase (Gene Fanavar, Iran) under the following conditions: initial denaturation step at 95°C for 5 minutes, followed by 35 cycles of denaturation at 94°C for 1 minute, annealing for 1 minute, and extension at 72°C for 1 minute. After the last cycle, the mixture was incubated at 72°C for 5 minutes. The amplification products were analyzed by electrophoresis on a 1.5% agarose gel. Images were obtained after staining of the gels with ethidium bromide and under an ultraviolet (UV) light imaging system.

4. Results

The results of the PCR assays showed specificity of primer pairs that were used for detection of the target bacteria. Accordingly, the species-specific primers provide a single PCR amplicon in the spiked stool samples (Figure 1). Analysis of culture method results for the detection of *C. jejuni* showed a sensitivity limit of 10⁴ CFU/g for the spiked stool samples. This detection level was as low as 10¹ CFU when the bacterium was directly inoculated on the culture medium from the PBS suspensions. PCR results showed a lower detection limit for the spiked stool samples (10² CFU/g). The validity of these results was confirmed by obtaining the same results for both of the studied strains in two separate tests. Analysis of the culture results for detection of *C. difficile* on selective medium showed its sensitivity for the detection of 10 organisms in 1 gram of stool sample. The PCR detection limit for this bacterium was 100 CFU/g; however, an increased detection limit of up to 10 genome copies per PCR reaction was obtained on prepared dilutions of genomic DNA. In the case of *Y. enterocolitica*, there was also discordance between results obtained by culture and those using the PCR method. In the culture method, *Y. enterocolitica* was detected in all dilutions of bacteria in PBS. In contrast, a lower limit of detection was observed via PCR (10⁴ CFU/g). The results of anaerobic culture for *C. perfringens* showed a detection limit of 2 × 10⁴ CFU/g that was similar to those were obtained by PCR assay results.

5. Discussion

The prevalence levels of *Campylobacter*, *Y. enterocolitica*, and *C. difficile* are about 10.8%, 1.2%, and 21%, respectively, in stool samples of patients with gastroenteritis in developing countries (28-30). These rates are lower than those reported for developed countries (4, 31). Despite differences in geographic area and culture in these countries, inaccurate results could be obtained due to the need for individual equipment and defined culture media for the detection of these pathogens in patients' stool samples. Because of their advantages, molecular tests are now widely used to detect these bacteria in clinical specimens. These tests are extremely useful diagnostic tools and are particularly valuable for the detection of infectious agents that are difficult to grow in conventional culture media. However, the presence of PCR inhibitors and variation of procedures that are used for sample preparation or extraction of nucleic acids affects their accuracy, precision, specificity, and sensitivity (23). The interpretation of results for these assays should be based on their limit of detection.

In this study, analysis of culture and PCR methods for the detection of *C. jejuni* showed a sensitivity limit of 10⁴ and 10² CFU/g, respectively, for the spiked stool samples. In a study by Singh et al. (32), higher sensitivity of PCR compared with culture method was indicated in the spiked fecal samples (Table 3). Our results showed lower detection limits for both the culture and PCR assays compared with those reported by Persson et al. (33) (pure culture, 10¹⁻² CFU; spiked stool, 10⁵ CFU/g). The obtained detection level for conventional PCR in our study was similar to those reported for *C. jejuni* and *C. coli* using the real-time PCR method (2.5 × 10² CFU/g of feces) (34). The incongruent results could be explained as relating to differences in the primer sequences and DNA extraction methods used in this study. Recovery of *C. jejuni* from stool samples may be affected by the types of culture media used. Potturi-Venkata et al. (35) showed a higher isolation rate for modified charcoal cefoperazone deoxycholate (mCCDA) compared with Brucella agar-based media for isolation of *Campylobacter* spp. from fecal samples (Table 3). *C. jejuni* is a microaerophilic bacterium that requires specific incubation conditions to grow in synthetic culture media. Although usage of an effective culture medium will improve the isolation rate of the bacterium, the need to provide microaerophilic conditions for its growth and supplements to prevent the growth of fecal microbiota are considered the main limitations of this method.

Alcohol pretreatment of stool specimens together with appropriate incubation time (up to 1 week) seems to be an effective method for the detection of *C. difficile* spores in the stool samples (7, 8); however, usage of other sensitive

Table 1. Primers Used in This Study

Bacterium	Target Gene	Primer	Sequence (5' to 3')	Amplicon Size (bp)	Tm °C	Reference
<i>Campylobacter</i> spp.	16S rRNA	C412F; C1228R	F: GGATGA-CACCTTTTCGGAGC	816	48	(24)
			R: CATTGTAGCACGT-GTGTC			
<i>Yersinia</i> spp.	16S rRNA	227Fmod; 669R	F: GTCTGGGCTTTGCTG-GTC	428 - 465	43	(25)
			R: GCGTCGTATTAG-CACCAACG			
<i>C. difficile</i>	<i>Cdd-3</i>	Tim6; Struppi6	F: TCC AATAATAAATTAG-CATTCC	622	54	(26)
			R: GGCATATACACG-TAATCCAGATA			
<i>C. perfringens</i>	16S rRNA	184-205; 441-462	F: AAAGATGGCAT-CATCATTCAC	279	50	(27)
			R: TACGTCAT-TATCTTCCCAAA			

Table 2. Detection Limits of *Campylobacter*, *Clostridium* and *Yersinia* spp. (CFU/gram stool) in the Culture and PCR Assays Using Spiked Stool Samples

	Limit of Detection, CFU/Gram Stool			
	<i>C. jejuni</i>	<i>C. difficile</i>	<i>C. perfringens</i>	<i>Y. enterocolitica</i>
Culture	10 ⁴	10	2 × 10 ⁴	10
PCR	10 ²	10 ²	2 × 10 ⁴	10 ⁴

Table 3. Comparison of the Culture and Molecular Assay Methods for Quantitative Detection of Fastidious Enteropathogens in the Spiked Human Stool Samples

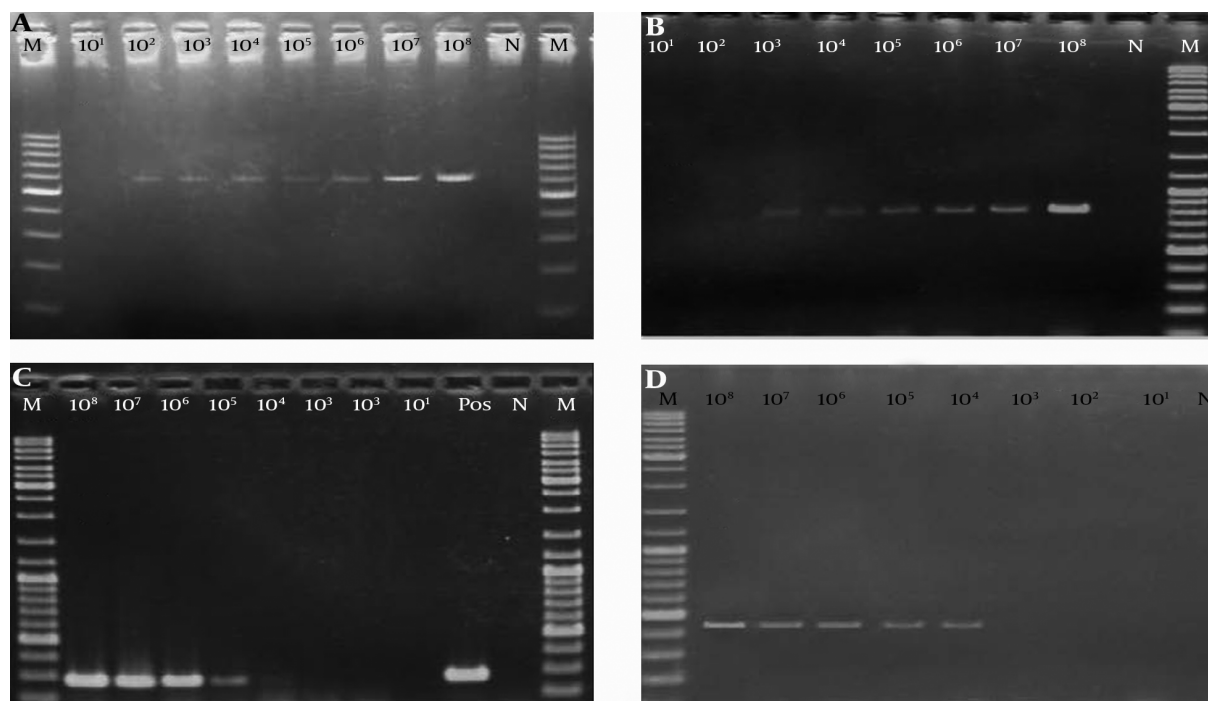
Bacterium	Method	Results, CFU g ⁻¹ /DNA Copy ^a	References
<i>Campylobacter</i> spp.	Culture and PCR	10 ⁴ /10 ²	This study
	Culture and PCR	10 ² /10 ²	(33)
	Real-time PCR	2.5 × 10 ²	(36)
	qPCR	10 ²	(37)
<i>Y. enterocolitica</i>	Culture and PCR	10/10 ⁴	This study
	Multiplex PCR	10 ⁵	(38)
	Real-time PCR	10 ²	(39)
	Culture and PCR	4 × 10 ² /4 × 10 ²	(40)
<i>C. difficile</i>	Culture and PCR	10/10 ²	This study
	Real-time PCR	5 × 10 ⁴	(41)
<i>C. perfringens</i>	Culture and PCR	2 × 10 ⁴ /2 × 10 ⁴	This study
	Multiplex PCR	10 ²⁻⁴	(42)

^aCFU/g, colony forming unit/gram stool; DNA copy of target bacterium per gram stool was represented for all the molecular assays.

and rapid tests is preferable. In our experiment, the PCR detection limit for this bacterium was 100 CFU/g. It seems that anaerobic culture has a much lower detection limit (10 CFU/g) than PCR assay for the detection of *C. difficile*. Belanger et al. (41) used real-time PCR assay for detection of

C. difficile, and their detection limit was estimated to be as high as 5 × 10⁴ CFU per gram of feces. Using the prepared dilutions of genomic DNA, an increased detection limit of up to 10 genome copies per PCR reaction was obtained in our experiment. According to these results, the incongruence of the PCR results compared with the culture results could be explained by the low yield of DNA that was extracted from the *C. difficile* spores in the spiked stool samples or the existence of mutations in the *cdd3* locus in the regions where our primers adhered. The existence of mutations was not supported by our data, since *cdd3* was detected in the DNA extracts of diluted DNA samples at the lower concentration. In a study by Luna et al. (43), where *tcdA* and *tcdB* were targeted in the spiked stool samples by real-time PCR, the lower limit of detection of *C. difficile* was 250 CFU/mL for *tcdA* and 500 CFU/ml for *tcdB*. These researchers similarly concluded that the sensitivity of the tests can only be increased in the more concentrated samples.

Discordance between results of culture and PCR methods was also determined in the case of *Y. enterocolitica*. This finding was supported by Weimer et al. (38), who used multiplex real-time PCR for the simultaneous detection of *Y. enterocolitica* and other bacteria in stool samples; they reported a sensitivity limit of 10⁵ CFU/g in their research. In

Figure 1. PCR ResultsA, *C. difficile*; B, *C. jejuni*; C, *C. perfringens*; D, *Y. enterocolitica*.

another study, detection limits of 10² CFU/mL and 10³ CFU/g for the pure culture and stool sample, respectively, were obtained using real-time PCR (39). However, Boyapalle et al. (40) reported a lower detection limit for PCR (4 × 10² CFU/g) compared with culture method (4 × 10³ CFU/g). The lower sensitivity of the PCR method compared with the culture method was also confirmed by the results of our assay using DNA extracts of the provided dilutions of *Y. enterocolitica* in PBS (71.4%, 10³ CFU/mL). In a study by Wan-net et al. (44), those primers that targeted *ail* and 16s rRNA genes showed a sensitivity of 100% (one genomic copy) in pure culture of *Y. enterocolitica*. Differences in the primers that target the *ompF* gene for PCR and the type of DNA extraction kit used in our experiment may explain these contradictory results. Re-analysis of the tests using different primers and extraction kits will provide more accurate data about their limits of detections.

C. perfringens is not only a member of human microbiota in the gastrointestinal tract, but it is also considered as a common cause of food poisoning in foodborne outbreaks (45). In general, detection of > 10⁶⁻⁸ CFU per gram of this bacterium in stool samples of patients with gastroenteritis is considered clinically important (42). Our results showed a similar detection limit (2 × 10⁴ CFU/g) for

both the culture and PCR methods. This amount was similar to those reported by Wise et al. (42) using a multiplex PCR assay. In their study, *C. perfringens* alpha and enterotoxin genes were targeted for detection of the bacterium in spiked fecal samples of domestic animals, and an average sensitivity of 10²⁻⁴ CFU/g was reported. Our results illustrated a correlation between the PCR assay and traditional culture method for the detection of *C. perfringens* in fecal spiked samples. Since the studied samples were subjected to heat treatment for the elimination of non-spore-forming bacteria (which may affect the germinating cells of *C. perfringens*), it seems that the detection limits of these tests are lower than 10³ CFU/g in human fecal samples.

The PCR results for all bacteria mentioned in this study were available on the same day as the assays were performed, whereas the culture results took 24 hours for *Y. enterocolitica* and 48 - 72 hours for *C. jejuni*, *C. difficile*, and *C. perfringens*. These results collectively showed that direct plating can be used successfully for the detection of anaerobic enteric bacterial pathogens (*C. difficile* and *C. perfringens*) and fastidious bacteria (*Yersinia* spp. and *Campylobacter* spp.) in human fecal samples when a bacterial load of greater than 10⁴ CFU/gram is present. The specified PCR assays showed acceptable results with respect to detec-

tion limits, which makes these methods especially suitable for rapid diagnostics of slow-growing bacteria in the fecal samples of infected patients. Improvements in the DNA extraction method and target sequences of the primers are needed to achieve more accurate results.

Footnotes

Authors' Contribution: Masoumeh Azimirad conceived of the work and conducted the study. Nastaran Farzi conceived of the work and agreed on all aspects of the research. Mohammad Hassan Shirazi conceived of the work, conducted the study, and agreed on all aspects of the research. Naser Ebrahimi Daryani conceived of the work, collected samples, and agreed on all aspects of the research. Mohammad Reza Zali conceived of the work, conducted the study, and agreed on all aspects of the research. Abbas Mirshafiey conceived of the work, conducted the study, and agreed on all aspects of the research. Seyed Saeed Eshraghi conceived of the work, conducted the study, and agreed on all aspects of the research.

Funding/Support: This study supported by the food- and waterborne diseases research center, Shahid Beheshti University, Tehran, Iran.

References

- Gerritsen J, Smidt H, Rijkers GT, de Vos WM. Intestinal microbiota in human health and disease: the impact of probiotics. *Genes Nutr*. 2011;**6**(3):209-40. doi: [10.1007/s12263-011-0229-7](#). [PubMed: [21617937](#)].
- Guarner F, Malagelada JR. Gut flora in health and disease. *Lancet*. 2003;**361**(9356):512-9.
- Hofreuter D, Tsai J, Watson RO, Novik V, Altman B, Benitez M, et al. Unique features of a highly pathogenic *Campylobacter jejuni* strain. *Infect Immun*. 2006;**74**(8):4694-707. doi: [10.1128/IAI.00210-06](#). [PubMed: [16861657](#)].
- Allos BM. *Campylobacter jejuni* Infections: update on emerging issues and trends. *Clin Infect Dis*. 2001;**32**(8):1201-6. doi: [10.1086/319760](#). [PubMed: [11283810](#)].
- Allos BM, Moore MR, Griffin PM, Tauxe RV. Surveillance for sporadic foodborne disease in the 21st century: the FoodNet perspective. *Clin Infect Dis*. 2004;**38** Suppl 3:115-20. doi: [10.1086/381577](#). [PubMed: [15095179](#)].
- Ashbolt NJ. Microbial contamination of drinking water and disease outcomes in developing regions. *Toxicology*. 2004;**198**(1-3):229-38. doi: [10.1016/j.tox.2004.01.030](#). [PubMed: [15138046](#)].
- Bartlett JG. Narrative review: the new epidemic of *Clostridium difficile*-associated enteric disease. *Ann Intern Med*. 2006;**145**(10):758-64. [PubMed: [17116920](#)].
- Leggett HC, Cornwallis CK, West SA. Mechanisms of pathogenesis, infective dose and virulence in human parasites. *PLoS Pathog*. 2012;**8**(2):1002512. doi: [10.1371/journal.ppat.1002512](#). [PubMed: [22359500](#)].
- Coker AO, Isokpehi RD, Thomas BN, Amisu KO, Obi CL. Human campylobacteriosis in developing countries. *Emerg Infect Dis*. 2002;**8**(3):237-44. doi: [10.3201/eid0803.010233](#). [PubMed: [11927019](#)].
- Olsen JE, Aabo S, Hill W, Notermans S, Wernars K, Granum PE, et al. Probes and polymerase chain reaction for detection of food-borne bacterial pathogens. *Int J Food Microbiol*. 1995;**28**(1):1-78. [PubMed: [8751091](#)].
- Sloan LM, Duresko BJ, Gustafson DR, Rosenblatt JE. Comparison of real-time PCR for detection of the *tcdC* gene with four toxin immunoassays and culture in diagnosis of *Clostridium difficile* infection. *J Clin Microbiol*. 2008;**46**(6):1996-2001. doi: [10.1128/JCM.00032-08](#). [PubMed: [18434563](#)].
- Oliver JD. The viable but nonculturable state in bacteria. *J Microbiol*. 2005;**43** Spec No:93-100. [PubMed: [15765062](#)].
- Talibart R, Denis M, Castillo A, Cappellet JM, Ermel G. Survival and recovery of viable but noncultivable forms of *Campylobacter* in aqueous microcosm. *Int J Food Microbiol*. 2000;**55**(1-3):263-7. [PubMed: [10791755](#)].
- 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**(Pt 7):617-22. doi: [10.1099/jmm.0.45607-0](#). [PubMed: [15184531](#)].
- Alvarez J, Sota M, Vivanco AB, Perales I, Cisterna R, Rementeria A, et al. Development of a multiplex PCR technique for detection and epidemiological typing of salmonella in human clinical samples. *J Clin Microbiol*. 2004;**42**(4):1734-8. [PubMed: [15071035](#)].
- Collins E, Glennon M, Hanley S, Murray AM, Cormican M, Smith T, et al. Evaluation of a PCR/DNA probe colorimetric membrane assay for identification of *Campylobacter* spp. in human stool specimens. *J Clin Microbiol*. 2001;**39**(11):4163-5. doi: [10.1128/JCM.39.11.4163-4165.2001](#). [PubMed: [11682549](#)].
- Wang G, Clark CG, Taylor TM, Pucknell C, Barton C, Price L, et al. Colony multiplex PCR assay for identification and differentiation of *Campylobacter jejuni*, *C. coli*, *C. lari*, *C. upsaliensis*, and *C. fetus* subsp. *fetus*. *J Clin Microbiol*. 2002;**40**(12):4744-7. [PubMed: [12454184](#)].
- Yang C, Jiang Y, Huang K, Zhu C, Yin Y. Application of real-time PCR for quantitative detection of *Campylobacter jejuni* in poultry, milk and environmental water. *FEMS Immunol Med Microbiol*. 2003;**38**(3):265-71. [PubMed: [14522462](#)].
- Clermont O, Bonacorsi S, Bingen E. Rapid and simple determination of the *Escherichia coli* phylogenetic group. *Appl Environ Microbiol*. 2000;**66**(10):4555-8. [PubMed: [11010916](#)].
- Stamper PD, Alcabasa R, Aird D, Babiker W, Wehrlin J, Ikpeama I, et al. Comparison of a commercial real-time PCR assay for *tcdB* detection to a cell culture cytotoxicity assay and toxigenic culture for direct detection of toxin-producing *Clostridium difficile* in clinical samples. *J Clin Microbiol*. 2009;**47**(2):373-8. doi: [10.1128/JCM.01613-08](#). [PubMed: [19073875](#)].
- Aichinger E, Schleck CD, Harmsen WS, Nyre LM, Patel R. Nonutility of repeat laboratory testing for detection of *Clostridium difficile* by use of PCR or enzyme immunoassay. *J Clin Microbiol*. 2008;**46**(11):3795-7. doi: [10.1128/JCM.00684-08](#). [PubMed: [18784320](#)].
- Jones DM, Sutcliffe EM, Curry A. Recovery of viable but non-culturable *Campylobacter jejuni*. *J Gen Microbiol*. 1991;**137**(10):2477-82. doi: [10.1099/00221287-137-10-2477](#). [PubMed: [1770362](#)].
- Burd EM. Validation of laboratory-developed molecular assays for infectious diseases. *Clin Microbiol Rev*. 2010;**23**(3):550-76. doi: [10.1128/CMR.00074-09](#). [PubMed: [20610823](#)].
- Onori M, Coltella L, Mancinelli L, Argentieri M, Menichella D, Villani A, et al. Evaluation of a multiplex PCR assay for simultaneous detection of bacterial and viral enteropathogens in stool samples of paediatric patients. *Diagn Microbiol Infect Dis*. 2014;**79**(2):149-54. doi: [10.1016/j.diagmicrobio.2014.02.004](#). [PubMed: [24656922](#)].
- Stenkova AM, Isaeva MP, Rasskazov VA. [Development of a multiplex PCR for detection of the *Yersinia* genus with identification of pathogenic species (*Y. pestis*, *Y. pseudotuberculosis*, *Y. enterocolitica*)]. *Mol Gen Mikrobiol Virusol*. 2008;**3**:18-23. [PubMed: [18756819](#)].
- Spigaglia P, Mastrantonio P. Molecular analysis of the pathogenicity locus and polymorphism in the putative negative regulator of toxin production (*TcdC*) among *Clostridium difficile* clinical isolates. *J Clin Microbiol*. 2002;**40**(9):3470-5. [PubMed: [12202595](#)].

27. Wu J, Zhang W, Xie B, Wu M, Tong X, Kalpoe J, et al. Detection and toxin typing of *Clostridium perfringens* in formalin-fixed, paraffin-embedded tissue samples by PCR. *J Clin Microbiol.* 2009;**47**(3):807-10. doi: [10.1128/JCM.01324-08](#). [PubMed: [19109478](#)].
28. Jalali M, Khorvash F, Warriner K, Weese JS. *Clostridium difficile* infection in an Iranian hospital. *BMC Res Notes.* 2012;**5**:159. doi: [10.1186/1756-0500-5-159](#). [PubMed: [22436392](#)].
29. Dallal MMS, Doyle MP, Rezadehbashi M, Dabiri H, Sanaei M, Modarresi S, et al. Prevalence and antimicrobial resistance profiles of *Salmonella* serotypes, *Campylobacter* and *Yersinia* spp. isolated from retail chicken and beef, Tehran, Iran. *Food Control.* 2010;**21**(4):388-92.
30. Feizabadi MM, Dolatabadi S, Zali MR. Isolation and drug-resistant patterns of *Campylobacter* strains cultured from diarrheic children in Tehran. *Jpn J Infect Dis.* 2007;**60**(4):217-9. [PubMed: [17642538](#)].
31. Ricciardi R, Rothenberger DA, Madoff RD, Baxter NN. Increasing prevalence and severity of *Clostridium difficile* colitis in hospitalized patients in the United States. *Arch Surg.* 2007;**142**(7):624-31. doi: [10.1001/archsurg.142.7.624](#). [PubMed: [17638799](#)].
32. Singh H, Rathore RS, Singh S, Cheema PS. Comparative analysis of cultural isolation and PCR based assay for detection of *campylobacter jejuni* in food and faecal samples. *Braz J Microbiol.* 2011;**42**(1):181-6. doi: [10.1590/S1517-83822011000100022](#). [PubMed: [24031619](#)].
33. Persson S, Olsen KE. Multiplex PCR for identification of *Campylobacter coli* and *Campylobacter jejuni* from pure cultures and directly on stool samples. *J Med Microbiol.* 2005;**54**(Pt 11):1043-7. doi: [10.1099/jmm.0.46203-0](#). [PubMed: [16192435](#)].
34. Leblanc-Maridor M, Beaudeau F, Seegers H, Denis M, Belloc C. Rapid identification and quantification of *Campylobacter coli* and *Campylobacter jejuni* by real-time PCR in pure cultures and in complex samples. *BMC Microbiol.* 2011;**11**:113. doi: [10.1186/1471-2180-11-113](#). [PubMed: [21600037](#)].
35. Potturi-Venkata LP, Backert S, Lastovica AJ, Vieira SL, Norton RA, Miller RS, et al. Evaluation of different plate media for direct cultivation of *Campylobacter* species from live broilers. *Poult Sci.* 2007;**86**(7):1304-11. [PubMed: [17575175](#)].
36. Banting GS, Braithwaite S, Scott C, Kim J, Jeon B, Ashbolt N, et al. Evaluation of Various *Campylobacter*-Specific Quantitative PCR (qPCR) Assays for Detection and Enumeration of *Campylobacteraceae* in Irrigation Water and Wastewater via a Miniaturized Most-Probable-Number-qPCR Assay. *Appl Environ Microbiol.* 2016;**82**(15):4743-56. doi: [10.1128/AEM.00077-16](#). [PubMed: [27235434](#)].
37. Orlofsky E, Benami M, Gross A, Dutt M, Gillor O. Rapid MPN-Qpcr screening for pathogens in air, soil, water, and agricultural produce. *Water Air Soil Pollut.* 2015;**226**(9):1-10.
38. Wiemer D, Loderstaedt U, von Wulffen H, Priesnitz S, Fischer M, Tannich E, et al. Real-time multiplex PCR for simultaneous detection of *Campylobacter jejuni*, *Salmonella*, *Shigella* and *Yersinia* species in fecal samples. *Int J Med Microbiol.* 2011;**301**(7):577-84. doi: [10.1016/j.ijmm.2011.06.001](#). [PubMed: [21855409](#)].
39. Zheng HX, Zhang MJ, Sun Y, Jiang B. [Detection of *Yersinia enterocolitica* in diarrhea stool by real-time PCR]. *Zhonghua Yi Xue Za Zhi.* 2006;**86**(32):2281-4. [PubMed: [17064577](#)].
40. Boyapalle S, Wesley IV, Hurd HS, Reddy PG. Comparison of culture, multiplex, and 5' nuclease polymerase chain reaction assays for the rapid detection of *Yersinia enterocolitica* in swine and pork products. *J Food Prot.* 2001;**64**(9):1352-61. [PubMed: [11563512](#)].
41. Belanger SD, Boissinot M, Clairoux N, Picard FJ, Bergeron MG. Rapid detection of *Clostridium difficile* in feces by real-time PCR. *J Clin Microbiol.* 2003;**41**(2):730-4. [PubMed: [12574274](#)].
42. Wise MG, Siragusa GR. Quantitative detection of *Clostridium perfringens* in the broiler fowl gastrointestinal tract by real-time PCR. *Appl Environ Microbiol.* 2005;**71**(7):3911-6. doi: [10.1128/AEM.71.7.3911-3916.2005](#). [PubMed: [16000804](#)].
43. Luna RA, Boyanton BL, Mehta S, Courtney EM, Webb CR, Revell PA, et al. Rapid stool-based diagnosis of *Clostridium difficile* infection by real-time PCR in a children's hospital. *J Clin Microbiol.* 2011;**49**(3):851-7. doi: [10.1128/JCM.01983-10](#). [PubMed: [21209161](#)].
44. Wannet WJ, Reessink M, Brunings HA, Maas HM. Detection of pathogenic *Yersinia enterocolitica* by a rapid and sensitive duplex PCR assay. *J Clin Microbiol.* 2001;**39**(12):4483-6. doi: [10.1128/JCM.39.12.4483-4486.2001](#). [PubMed: [11724866](#)].
45. Scallan E, Hoekstra RM, Angulo FJ, Tauxe RV, Widdowson MA, Roy SL, et al. Foodborne illness acquired in the United States-major pathogens. *Emerg Infect Dis.* 2011;**17**(1):7-15. doi: [10.3201/eid1701.P11101](#). [PubMed: [21192848](#)].