JOURNAL OF CLINICAL MEDICINE OF KAZAKHSTAN (E-ISSN 2313-1519)
Original Article DOI: https://doi.org/10.23950/jcmk/11937

The 16s ribosomal ribonucleic acid microorganisms' detection in mesenteric lymph nodes by a polymerase chain reaction in view of colorectal cancer

Alina Ogizbayeva[1], Yermek Turgunov[1], Irina Kadyrova[2], Kayrat Shakeyev[1], Svetlana Kolesnichenko[2], Miras Mugazov[3], Ilshat Moldozhanov[3]

[1]Department of Surgical Diseases, Karaganda Medical University, Karaganda, Kazakhstan
[2]Collective Use Laboratory of the Research Centre, Karaganda Medical University, Karaganda, Kazakhstan
[3]Department of Anesthesiology, Resuscitation and Emergency Medical Care, Karaganda, Kazakhstan

Received: 2022-01-04.
Accepted: 2022-02-26

J Clin Med Kaz 2022; 19(2):38-42

Corresponding author:
Alina Ogizbayeva.
E-mail: eleusizova.a@kgmu.kz;
ORCID: 0000-0003-1006-1870

Abstract

Objective: This study proposes a method to detect 16s rRNA microorganisms in mesenteric lymph nodes (MLN) using a polymerase chain reaction (PCR) in patients with colorectal cancer (CRC).

Material and methods: To quantify the presence of microorganisms in MLN, it is proposed to determine the dependence of the accumulated amplification products on the number of colony-forming units of bacteria (CFU/ml). The pure culture of Escherichia coli, GFP 6 serotype of biotype 1 (ATCC® 25922GFP™) with the CFU values from 102 to 108 (group 1) as well as the mixtures of E.coli with CFU/ml from 102 to 108 with the MLN tissues (group 2) were calibrated. The third group consisted of the MLN patients (60 people) with CRC without bowel obstruction. The 16s rRNA bacteria in MLN was detected by using real-time PCR by the BIO-RAD CFX96 amplifier.

Results: To assess the dependence of the bacteria's CFU/ml logarithm on the value of the threshold cycle amplification, a model was developed in the form of an equation. The amplification curves, threshold cycle values, and PCR efficiency differ from the first two groups. This can be due to the presence of DNA amplification-inhibiting compounds as well as the non-specific binding of MLN primers to DNA. Therefore, a mathematical model of the second group (suspension of E.Coli and MLN) was used to study the translocation of microorganisms in MLN. According to the developed mathematical model, depending on the values of the threshold amplification cycles, the positive PCR result in the study group (patients with CRC) was detected in 15 patients (25%). At the same time, the level of CFU/ml with bacterial translocation in MLN does not exceed 104.

Conclusion: The developed method allows to determine the microbial DNA in MLN quantitatively in a wide range of its concentrations (102 to 108 CFU).

Key words: bacterial translocation, gut microbiota, intestinal barrier, colorectal cancer, PCR, 16s rRNA, mesenteric lymph nodes

Introduction

Colon and rectal cancer (CRC) ranks third in prevalence among all diagnosed malignancies [1]. Over the past 20 years in Kazakhstan, CRC has moved up in the structure of cancer incidence from 6th to the 3rd place [2]. It is the fourth leading cause of death due to cancer in the world [3, 4]. As of today, the rates of postoperative infectious and inflammatory complications remain high. These are one of the main causes of death in patients with cancer [4,5]. In case of CRC, there are disturbances in the microbiota of the large intestine as well as the violations of the microcirculation of the intestinal wall and the

intestinal barrier. These changes lead to the so-called bacterial translocation through the damaged intestinal wall into the mesenteric lymph nodes (MLN) and further into the systemic circulation, as a result of which postoperative infectious and inflammatory complications may occur [6].

To identify bacterial translocation in MLN, microbiological methods are used. These include the isolation of a pure culture of bacteria, yet this technique is time-consuming (it takes an average of 3-7 days). Besides, not all bacteria can be cultured in the microbiological media [7]. Another detection method of bacterial translocation in MLN is the use of radioactively labeled microorganisms. From the point of view of ethical standards, this technique is applicable only in the experiment. Recently, the technique for determining bacterial DNA in MLN, namely 16S rRNA (ribosomal ribonucleic acid), by using a polymerase chain reaction (PCR) in real-time (Real-time PCR) has become relevant. The 16S rRNA gene contains some hypervariable regions unique to each microorganism and some "rigid" regions common to all microorganisms. Therefore, there are universal primers that bind to the known common gene sequences of most bacteria. This is a relatively recently proposed method in which most pathogenic bacteria can be detected using universal primers [8].

The team of the authors has proposed a method for detecting 16S rRNA microorganisms in mesenteric lymph nodes by using a polymerase chain reaction in the patients with colon and rectal cancer.

Material and methods

To quantify the presence of microorganisms in MLN, it is proposed to determine the dependence of the accumulation of amplification products on the number of colony-forming units (CFU) of bacteria per milliliter (CFU/mL). Escherichia coli (E. coli) GFP 6 serotype 1 (ATCC® 25922GFP™) with CFU/mL values from 102 to108 as well as the mixtures of E. coli with CFU/mL from 102 to 108 with MLN tissues were calibrated to the pure culture.

The first group consisted of the pure culture of E. coli samples with 102, 104, 106, 108 CFU/mL in saline (0.9% NaCl solution) – 5 samples of each CFU/mL value, totally comprising 20 samples.

The second group consisted of the MLN samples with a suspension of E. coli with 102, 104, 106, 108 CFU/mL – 5 samples of each CFU/mL value (subgroups), a total of 20 samples.

The third group consisted of the MLN patients (60 people) with colorectal cancer without bowel obstruction. The operating surgeon performed a MLN sampling in sterile conditions during surgery after resection of the intestine from the mesentery of the gross specimen. MLN was placed in a sterile tube without any fillers. The MLU specimen was stored in the refrigerator (+4°C to +8°C) up to the moment of transportation (for a maximum period of up to 12 hours). The collected material was transported strictly vertically in a special container with cooling elements at a temperature of +2°C to +8°C for 6 hours.

The DNA was extracted by the GeneJET Genomic DNA Purification Kit manufactured by Thermo Fisher Scientific, USA, in accordance with the manufacturer's instructions. The samples of MLN weighing up to 20 mg (pure, bacterial-free and with added 102 to 108 bacteria) were placed in the microcentrifuge tubes with the addition of 180 µL Digestion Solution and 20 µL of Proteinase K solution. The tubes were shaken thoroughly by Vortex and incubated at 56°C. The samples had been shaken periodically by Vortex until the tissues were completely lysed. Furthermore, 20 µL of RNAse A solution was added, followed by a 10 min incubation at room temperature. 200 µL of lysing solution and 400 µL of 50% ethanol were added afterwards. After each step, the sample was thoroughly mixed by using Vortex. The mixture was then transferred to special spin columns with a test tube for collection and centrifuged for 1 min at 6.000 x g. After each centrifugation, the spin column was placed in a new test tube for collection. The samples were then washed with 500 µL of wash buffer I and centrifuged at 8.000 x g for a minute. The second stage of washing was carried out by adding 500 µl of wash buffer II with centrifugation at maximum speed for 3 minutes. The spin column was then placed in a sterile microcentrifuge tube and 200 µL of elution buffer was added, followed by incubation for 2 min at room temperature and centrifugation for 1 min at 8,000 x g.

The obtained DNA was immediately used for PCR diagnostics. The 16s rRNA bacteria in MLN was detected by using real-time PCR and BIO-RAD CFX96 amplifier. For this purpose, a reaction mixture was prepared, which consisted of:

1) 4 µL of the DNA sample under test;

2) 1 µL of 16s rRNA forward and reverse primers (U16SRT-F FACTCCTACGGGAGGGAGGCAGGT and U16SRT-R TATTACCGCGGCTGCTGGGC);

3) 10 µL of the Master Mix Maxima SYBR Green reaction mixture;

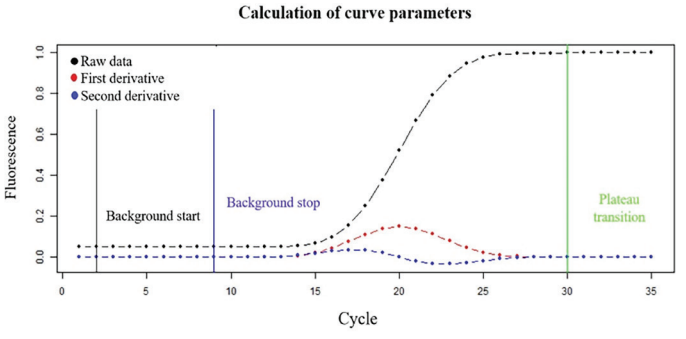
4) 4 µL of nuclease-free water.

Afterwards, the samples were loaded into the tablet of the BIO-RAD CFX96 amplifier (refer to the Figure 1), and amplified under the following parameters:

- denaturation at 95°C for 10 minutes;
- "annealing" and elongation – 40 cycles at 95°C for 15 seconds and 40 cycles at 62°C for 60 seconds.

After PCR amplification, the fluorescence values for the three groups were imported into R statistics (v.3.6.3) for further analysis. The qpcR and pcr packages were used to construct the sigmoid curves (Figure 1) [9]. To assess the dependence of the CFU/mL logarithm on the value of the threshold amplification cycle, a model was developed in the form of an equation.

Figure 1 - Amplification based on a sigmoid model with 4 parameters and calculated threshold cycles based on a derivative nonlinear model as well as the efficiency of amplification. First derivative – red line, second derivative – blue line, amplification graph – black line



Results and discussion

Figure 2 shows the curve of the PCR standards of the first calibration group (suspension of E. coli in saline), while Figure 3 demonstrates the second group of calibration (suspension of E. coli with tissues of MLN).

Using the average values of the threshold cycles (Ct) of the first and second groups (Tables 1 and 2), the graphical curves of the PCR standards were drawn up in the subgroups with different

Figure 2 - Amplification of E. coli samples with 102, 104, 106,108 CFU/mL in saline

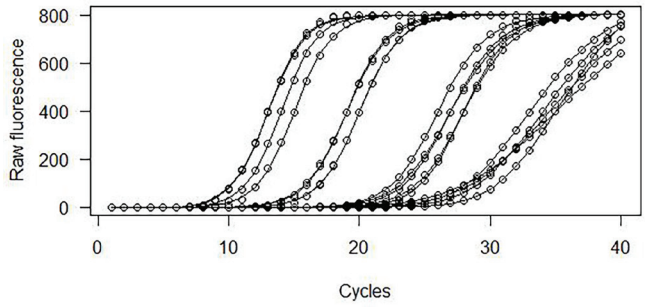


Figure 3 - Amplification of E. coli samples with 102, 104, 106,108 CFU/mL with mesenteric lymph nodes tissues

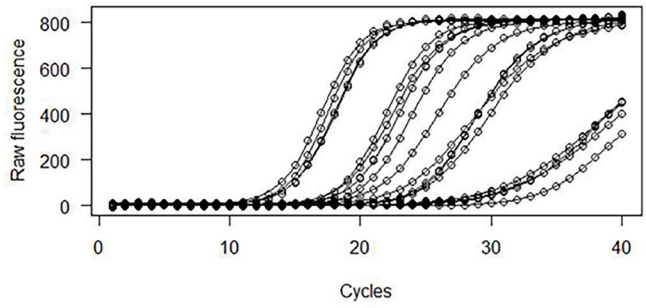


Table 1 Average values of threshold cycles in the E.coli group of 102, 104, 106,108 CFU/mL in saline

Subgroups, CFU/mL	n	Lg (CFU/mL)	Ct
102	5	2	34.885 ± 0.986
104	5	4	27.024 ± 1.086
106	5	6	19.694 ± 0.475
108	5	8	13.836 ± 0.639

The results are expressed as mean and standard deviation and number. CFU, colony-forming unit; Lg (CFU/mL) - logarithm of colony-forming units of bacteria per milliliter; Ct, cycle threshold.

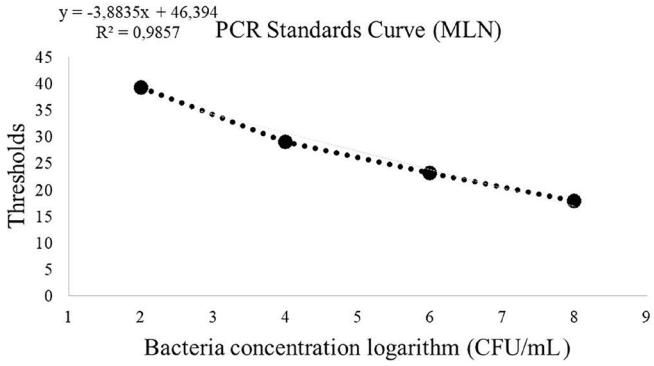
Table 2 Average values of threshold cycles in group E. coli of 102,104, 106, 108 CFU/mL with MLN tissue

Subgroups, CFU/mL	n	Lg (CFU/mL)	Ct
102	10	2	39.235 ± 0.87
104	10	4	29.002 ± 0.89
106	10	6	23.211 ± 1.11
108	10	8	17.899 ± 1.3

The results are expressed as mean and standard deviation and number. CFU - colony-forming unit; Lg (CFU/mL) - logarithm of colony-forming units of bacteria per milliliter; Ct - cycle threshold.

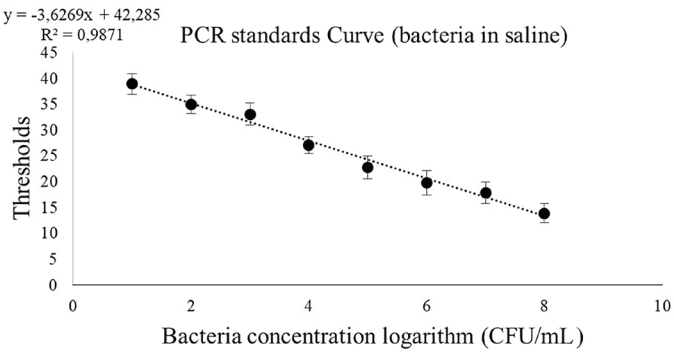
CFU/mL (from 108 to 102 CFU/mL). A model was developed in the form of an equation to assess the dependence of the CFU/mL logarithm on the value of the threshold amplification cycle (Figures 4 and 5).

Figure 4 - Polymerase chain reaction (PCR) standards Curve in group E. coli of 102,104, 106, 108 CFU/mL with mesenteric lymph nodes tissue (MLN)



The amplification curves, threshold cycle values and PCR efficiency differ between the two groups. This can be due to the presence of DNA amplification-inhibiting compounds as well as the non-specific binding of MLN primers to DNA. Some

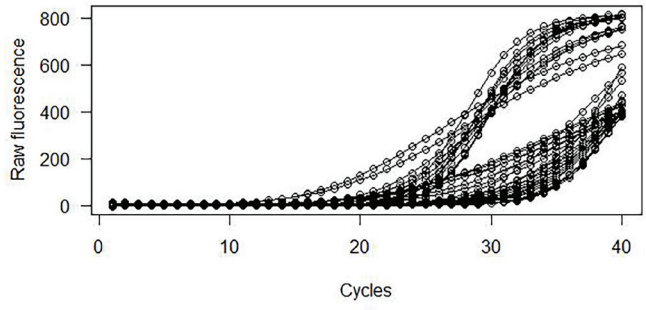
Figure 5 - Polymerase chain reaction (PCR) standards Curve in group E. coli of 102, 104, 106, 108 CFU/mL in saline



researchers reported that 16s rRNA primers align to a region within the human mitochondrial DNA and it can be amplified by this primers when human DNA is overwhelming [10, 11]. Therefore, to study the translocation of microorganisms in MLN, a mathematical model of the second calibration group (suspension E. coli and MLN) was used.

In accordance with the developed mathematical model, depending on the values of the threshold cycles amplification, the positive PCR result in the study group (patients with CRC) was found in 15 patients (25%). At the same time, the level of CFU/mL with bacterial translocation in MLN does not exceed 104 (Figure 6).

Figure 6 - Amplification of mesenteric lymph nodes tissue samples



Bacterial translocation (BT) to the lymph nodes occurs because a large area of intestinal mucosa is drained by lymphatic capillaries, each of which passes into larger lymphatic vessels and then to the lymph nodes [12]. Number of researches support the view that BT is associated with an increase in postoperative infectious complications and septic morbidity, as BT can induce an enhanced immune response and ultimately lead to systemic inflammatory responses [13, 14]. For example, Takashi Mizuno

et al. in patients after hepatectomy for biliary malignancies and Eiji Nishigaki et al. in patients after esophagectomy, reported that the detection of bacterial 16 s rRNA in MLN, was strongly associated with the occurrence of postoperative infectious complications [15, 16]. In this two studies bacterial 16s rRNA was detected in 37.3% MLN (19 of 51 cases) and in 56% MLN (10 of 18 cases), respectively. There are also several studies of 16s rRNA in MLN in patients with inflammatory bowel disease, where 16s rRNA was found in no more than 40% of MLN. But the sample size in these studies was small (20 patients each) and to determine the sensitivity of the PCR, researchers amplified ovine lymph nodes spiked with 103 CFU/mL, but not 102 CFU/mL, bacterial cells [12, 17]. Studies on the detection of bacteria in the MLN in colorectal cancer were carried out, but they used conventional culture-based methods, while in present study was used the novel method for determining bacterial 16s rRNA.

Because culture-based studies are limited and have only identified 20% of the bacteria present, detection of bacterial 16s rRNA is widely used. Today, the research on human microbiota has focused on material such as oral or vaginal swabs, feces that have a large number of bacterial populations and few human DNA [18]. Bacteria from MLN more difficult to assess by standard PCR due to small amount of bacteria and relatively large amount of human DNA.

Also, today optimal PCR methods for analysis of low biomass microbiota samples like MLN has not been developed. Remy Villette et al. tested the lower bacterial concentration required to perform 16S rRNA gene analysis: microbiota community standard and low biomass samples (108, 107, 106, 105 and 104 CFU/mL) from two healthy donor stools were employed to assess optimal sample processing for 16s rRNA gene analysis. Using the improved protocol they report a lower limit of 106 bacteria per sample for robust and reproducible microbiota analysis [19]. Studies to determine the lowest concentration of bacteria in the lymph nodes, necessary for the analysis of the 1s rRNA gene, have not been previously performed. In present study mathematical model depending on the values of the threshold cycle's amplification, was developed and the lower limit of bacteria in the lymph nodes was 104 CFU/mL.

Conclusion

The developed method allows to determine the microbial DNA in MLN quantitatively in a wide range of its concentrations (CFU/mL from 102 to 108). It was found that the level of CFU/mL in the event of CRC with the bacterial translocation in MLN does not exceed 104. Detection bacterial 16s rRNA gene in MLN, and other human tissues with low biomass microbiota samples are receiving increasing attention. Therefore, a more in-depth study of this problem and the development of an optimal standardization for this analysis are needed.

Ethical aspects: The study was conducted in accordance with the Ethical Guidelines for Medical Research with Human Subjects as established by the Ministry of Health of the Republic of Kazakhstan and with the guidelines outlined in the Helsinki Declaration and its amendments. This study was approved by the Ethics Committee of the NJSC "Karaganda Medical University" (Protocol No.6 with the assigned number No.30). An informed consent was obtained from all participants included in the study.

Disclosures: There is no conflict of interest for all authors.

Acknowledgements: None.

Funding: This research has been funded by the Science Committee of the Ministry of Education and Science of the Republic of Kazakhstan (Grant No. AP09260597).

References

1. Pisano M, Zorcolo L, Merli C, et al. 2017 WSES guidelines on colon and rectal cancer emergencies: obstruction and perforation. *World J Emerg Surg*. 2018; 13(1):36. https://doi.org/10.1186/s13017-018-0192-3
2. Nurshabaeva AE, Dauletkalieva ZA. Analysis of population coverage by screening studies for the early detection of colorectal cancer. *Astana medicine journal*. 2018; 4(98):154-159.
3. Catena F, De Simone B, Coccolini F, et al Bowel obstruction: a narrative review for all physicians. *World J Emerg Surg*. 2019; 14(1):20. https://doi.org/10.1186/s13017-019-0240-7
4. Zembower TR. Epidemiology of infections in cancer patients. *Cancer Treat.Res*. 2014; 161:43–89. https://doi.org/10.1007/978-3-319-04220-6_2
5. Galloway-Peña J, Brumlow C, Shelburne S. Impact of the Microbiota on Bacterial Infections during Cancer Treatment. *Trends Microbiol* 2017; 25(12):992–1004. https://doi.org/10.1016/j.tim.2017.06.006
6. Schietroma M, Pessia B, Colozzi S, et al. Septic complications after resection for middle or low rectal cancer: role of gut barrier function and inflammatory serum markers. *Dig Surg*. 2017; 34(6):507–517. https://doi.org/10.1159/000475847
7. Oliver JD. The viable but nonculturable state in bacteria. J Microbiol. 2005; 43:93–100. PMID: 15765062.
8. Ames NJ, Ranucci A, Moriyama B, Wallen, GR. The Human Microbiome and Understanding the 16S rRNA Gene in Translational Nursing Science. *Nurs Res*. 2017; 66(2):184–197. https://doi.org/10.1097/NNR.0000000000000212
9. Ritz C, Spiess A-N. qpcR: an R package for sigmoidal model selection in quantitative real-time polymerase chain reaction analysis. *Bioinformatics*. 2008; 24(13):1549–1551. https://doi.org/10.1093/bioinformatics/btn227
10. Walker SP, Barrett M, Hogan G et al. Non-specific amplification of human DNA is a major challenge for 16S rRNA gene sequence analysis. Sci Rep. 2020; 10(1). https://doi.org/10.1038/s41598-020-73403-7
11. Earl JP, Adappa ND, Krol J, et al. Species-level bacterial community profiling of the healthy sinonasal microbiome using Pacific Biosciences sequencing of full-length 16S rRNA genes. *Microbiome*. 2018; 6(1):190. https://doi.org/10.1186/s40168-018-0569-2
12. Kiely CJ, Pavli P, O'Brien CL. The microbiome of translocated bacterial populations in patients with and without inflammatory bowel disease. *Intern Med J*. 2018; 10:44. https://doi.org/10.1111/imj.13998
13. Deitch EA. Bacterial translocation or lymphatic drainage of toxic products from the gut: what is important in human beings? *Surgery*. 2002; 131(3):241– 244. https://doi.org/10.1067/msy.2002.116408

14. Gatt M, Reddy BS, MacFie J. Review article: bacterial translocation in the critically ill—evidence and methods of prevention. *Aliment Pharmacol Ther.* 2007; 25(7):741–57. https://doi.org/10.1111/j.1365-2036.2006.03174.x
15. Mizuno T, Yokoyama Y, Nishio H. Intraoperative Bacterial Translocation Detected by Bacterium-Specific Ribosomal RNA-Targeted Reverse-Transcriptase Polymerase Chain Reaction for the Mesenteric Lymph Node Strongly Predicts Postoperative Infectious Complications After Major Hepatectomy for Biliary Malignancies. *Annals of Surgery.* 2010; 252(6):1013-1019. https://doi.org/10.1097/SLA.0b013e3181f3f355
16. O'Brien CL, Pavli P, Gordon DM, Allison GE. Detection of bacterial DNA in lymph nodes of Crohn's disease patients using high throughput sequencing. *Gut*. 2014; 63:1596-606. https://doi.org/10.1136/gutjnl-2013-305320
17. Nishigaki E, Abe T, Yokoyama Y. The Detection of Intraoperative Bacterial Translocation in the Mesenteric Lymph Nodes Is Useful in Predicting Patients at High Risk for Postoperative Infectious Complications After Esophagectomy. *Annals of Surgery*. 2014; 259(3). https://doi.org/10.1097/SLA.0b013e31828e39e8
18. Yu G, Fadrosh D, Goedert JJ et al. Nested PCR Biases in Interpreting Microbial Community Structure in 16S rRNA Gene Sequence Data sets. *PlosOne*. 2015; 10(7):e0132253. https://doi.org/10.1371/journal.pone.0132253
19. Villette R, Autaa G, Hind S, et al. Refinement of 16S rRNA gene analysis for low biomass biospecimens. *Sci Rep*. 2021; 11:1074. https://doi.org/10.1038/s41598-021-90226-2