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Research Article

Study of the Nature of Contamination of Raw Milk with Bacteria of the Genus Campylobacter Using Traditional Microbiological Methods and Quantitative PCR Analysis

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Abstract

Introduction: *Campylobacter* gastroenterocolitis is the most common foodborne acute infectious zoonotic disease. One of the important factors in the transmission of infection is contaminated dairy products, therefore, the assessment of contamination of dairy raw materials with *Campylobacter* is necessary to develop effective measures to suppress the growth of the pathogen and ensure the safety of products.

Aim of the Study: The aim of the study was to study the microbial background of raw milk samples and the nature of their contamination with thermophilic bacteria of the genus *Campylobacter*.

Materials and Methods: 60 samples of raw milk from the central regions of the Russian Federation and 48 samples of raw milk experimentally infected with *Campylobacter* were studied. To assess microbial contamination of milk, the amount of extraneous microflora, including coliform bacteria (BCB), was determined. The identification and determination of the number of bacteria of the genus *Campylobacter* was carried out by cultural methods in comparison with quantitative analysis by the method of polymerase chain reaction (PCR analysis). For PCR, primers were used that detected the species-specific sequence of 16s rRNA of *C. jejuni*, the presence of the cytolethal toxin cdtB gene and the ciaB invasion gene.

Results and Discussion: A significant part of the raw milk samples examined (31.6%) were characterized by high levels of microbial contamination, exceeding 10^6 CFU/cm^3. Gram-negative bacteria are the dominant type of bacterial microflora, their levels were comparable to the revealed values of the total number of microorganisms. BGKP were found in all the samples studied, their number in 90% of the samples reached 10^5 CFU/cm^3 and in some samples - 10^7 CFU/cm^3. Detection rate of *Campylobacter* spp. in raw milk was 8.3% and their number ranged from 0.1 to 100 CFU/cm^3 (on average 2.0 x 10 CFU/cm^3). All the isolated *Campylobacter* strains were identified as *C. jejuni* by a complex of phenotypic characters. Comparative analysis of contamination of raw milk with *Campylobacter* by RT-PCR was carried out. Most of the samples (over 60%) were positive for the genome-specific genomic sequence of 16SrRNA, while they were characterized by the highest values of the total bacterial contamination and the number of BHCPs. The use of a multi-primer approach (simultaneous testing for the presence of 16SrRNA and the cytolethal toxin cdtB gene of *C. jejuni*) reduced the number of positive cases of *Campylobacter* DNA detection to 16.6%, which suggests that the cdtB gene is more informative in relation to the detection of viable, including uncultured, cells. with toxigenicity. A tentative assessment of the results in a quantitative format indicated high detectable levels of *Campylobacter* bacteria - 10^4 - 10^6-5 genomic equivalents/cm^3, which indicates the possible presence of viable *Campylobacter* cells in the test material with a significantly higher frequency than that established by culture.

Conclusion: Microbiological methods of inoculation at low levels of contamination with *Campylobacter* do not ensure their reliable detection due to massive contamination of raw materials with extraneous microflora. *Campylobacter* spp. were detected by the culture method in 8.3% of cases, while the use of multi-primer PCR analysis with the cdtB and ciaB genes makes it possible to double the detection of *C. jejuni* in raw milk samples.

Keywords: *Bacteria of the Genus Campylobacter; Raw Milk; PCR Analysis; Microbial Contamination*

Introduction

Studying the sources and frequency of isolation of pathogenic microorganisms, as well as the conditions for their survival and development in food is of great importance for ensuring consumer safety. Among bacterial foodborne infections, diseases caused by pathogens of the genus *Campylobacter* are the most widespread and difficult to overcome in the system of food safety and prevention of Campylobacteriosis [1-3]. Reliable identification of the sources of contamination with *Campylobacters* is necessary for the creation of methods for controlling these microorganisms in food, the development of effective technological regimes for suppressing the growth or destruction of contaminants and ensuring the specified microbiological quality of products.

Considering the wide prevalence of bacteria of the genus *Campylobacter* in nature and the variety of sources of contamination, much attention is paid at the present stage to the frequency of detection of these microorganisms in various objects, including on the surfaces of equipment and inventory of food industry enterprises. Despite the fact that the main route of *Campylobacter* ingestion into the human body is the consumption of contaminated meat products (especially poultry meat), the use of contaminated unpasteurized milk or dairy products produced from it remains one of the important factors in the transmission of infection [4-8].

Microorganisms enter food both from internal and external sources, with which the product can come into contact during the entire manufacturing period and until the moment of consumption. Sources of contamination of products of animal origin are skin, hair and wool, mucous membranes, gastrointestinal and urogenital tract, respiratory tract, mammary gland ducts and teat ducts of the udder of dairy animals. Diseases of animals and birds (intestinal infections, inflammation of the respiratory tract and urinary system, mastitis in cows) increase the frequency of excretion of bacterial pathogens. Violation of sanitary requirements during the slaughter of animals leads to contamination of the surfaces of carcasses and animal organs with *Salmonella*, *Campylobacter* and *Listeria* [9]. At the same time, the level of contamination of unprocessed livestock products is directly dependent on the intensity of infection and the degree of bacterial carriers of birds and animals.

Considering the main sources of animal origin, it should be recognized that the most likely reason for the spread of pathogens is the fecal route of infection of animal raw materials, water, soil and other environmental objects, from which microorganisms can enter food [10]. Fecal contamination is associated with the widespread occurrence of pathogenic zoonotic bacteria *Campylobacter jejuni* in the environment. Fecal contamination in some cases explains the ingestion of *Campylobacter* in raw milk (Table 1). It should be noted that the frequency of contamination of milk by *C. jejuni* is higher than that of other pathogens.

Causative agent	% detection
Campylobacter jejuni	9,2
Salmonella bacteria	6,1
Yersinia enterocolitica	6,0
Listeria monocytogenes	4,6
Enterohemorrhagic *E. coli* (EHEC)	3,8

Table 1: *Detection of emergent zoonotic contaminants in raw milk [11].*

Nevertheless, the conditions for the entry of bacteria of the genus *Campylobacter* into raw milk and the nature of contamination with these pathogens of dairy raw materials used for the production of various types of food products have been insufficiently studied [12-14].

Purpose of the Study

The purpose of this work is to study the microbial background of various raw milk samples and to identify the possible presence of *Campylobacter* in them.

Raw milk studies included the identification and counting of thermophilic *Campylobacter*, total bacterial contamination and levels of Gram-negative *Enterobacteriaceae*, including coliform bacteria (coliform bacteria). The objectives of the study also included an assessment of the species belonging of the isolated *Campylobacter* spp.

Materials and Methods

In the work, 60 samples of raw milk from the central regions of the Russian Federation and 48 experimentally infected samples of raw milk were studied.

The studies were carried out in accordance with GOST 32901-2013 "Milk and dairy products. Methods of microbiological analysis", MUK 4.2.2321-08 "Methods for the determination of bacteria of the genus *Campylobacter* in food" and GOST ISO 10272-1-2013 "Microbiology of food and feed for animals. Methods for the detection and enumeration of *Campylobacter* spp. Part 1: Method of detection". For inoculation of milk samples for the presence of bacteria of the genus *Campylobacter*, culture media containing antibiotics and aerotolerant additives were used, including Bolton broth, Preston's blood agar, modified carbon sodium deoxycholate agar (mCCD agar), and blood agar. Subculture on the surface of agar selective media and incubation was carried out in a microaerophilic atmosphere (10% CO_2, 5% O_2, 85% N_2) at 41.5 ± 0.5°C. Typical colonies grown on the surface of selective agar were identified by testing them by a combination of cultural, morphological and biochemical traits that determine their belonging to the *Campylobacter* bacteria.

Detection and quantitative determination of bacteria of the genus *Campylobacter* by real-time polymerase chain reaction (PCR) (RT-PCR) was carried out in accordance with GOST R 57989-2017 "Specialized food products. Methods for detecting pathogenic microorganisms based on polymerase chain reaction". For PCR, primers were used that detected the species-specific 16s rRNA sequence for *C. jejuni*, the presence of the gene for subunit B of the cytolethal toxin cdtB, and the gene for invasion ciaB. Genomic DNA was extracted using the NucliSens easyMag automated system (BioMerieux, France). For the quantitative determination of *C. jejuni*, calibration curves were constructed using test data as 10-fold dilutions of 4 test strains of *C. jejuni* as calibrators (See figure 1).

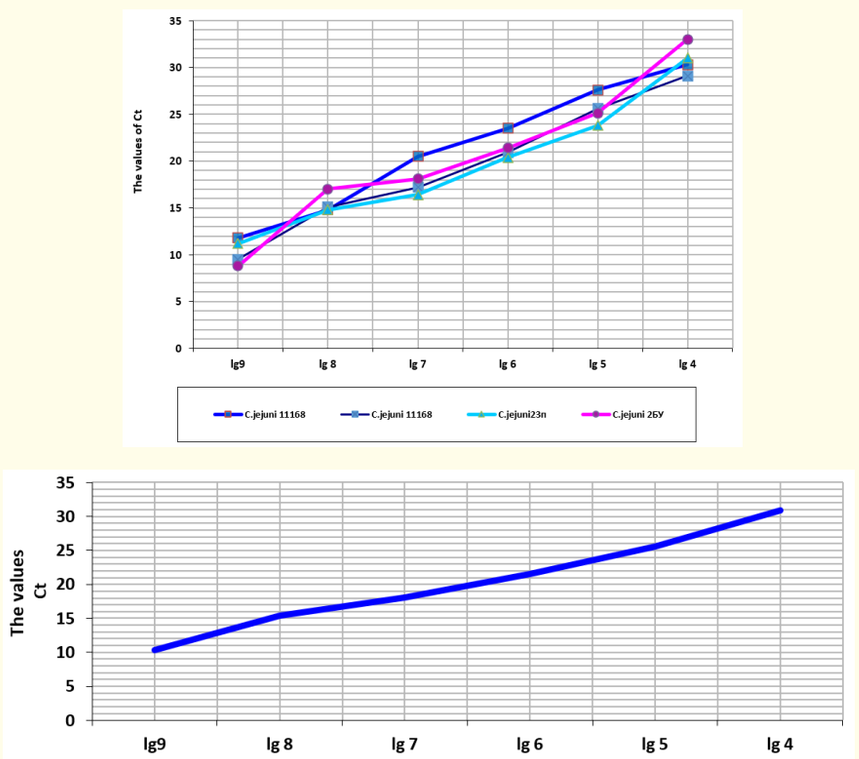

Figure 1: *Calibration curve for determining the amount of C. jejuni by CT values for PCR products of test strains (above - for four strains; below - average values).*

The number of *Campylobacter* DNA copies in the studied samples was estimated by quantitative RT-PCR. Amplification curves were analyzed using the ABI PRISM 7500 RealTime PCR system software (Applied Biosystems/Thermo Fisher Scientific, USA).

The total number of mesophilic aerobic and facultative anaerobic microorganisms (KMAFAnM) was determined according to GOST 10444.15-94 "Food products. Methods for determining the amount of mesophilic aerobic and facultative anaerobic microorganisms", BGKP - according to GOST 31747-2012 "Food products. Methods for detection and quantity determination coliform bacteria (coliform bacteria)".

Statistical processing of the results was carried out using the Excel and SPSS 18.0 software packages.

Results and Discussion

The analysis of experimental data showed that a significant part (31.6%) of the studied samples of raw milk is characterized by high levels of bacterial contamination - 10^6 CFU/cm^3 and more (Table 2). Assessment of milk contamination with coliform bacteria indicates that this group of microorganisms is dominant, its levels are comparable with the total amount of QMAFAnM. BGKP were found in 100% of samples, while the content of coliforms in the studied milk samples reached 10^7 CFU/cm^3 (on average - lg2.9 ± 0.2, median - lg3.0). Against the background of such a high microbial contamination, the number of positive samples in which bacteria of the genus *Campylobacter* were detected was 8.3%, which indicates a rather high frequency of their detection in raw milk. *Campylobacter* spp. in the examined samples ranged from 0.1 to 100 CFU/cm^3 (on average 2.0 x 101 CFU/cm^3). The isolated *Campylobacter* strains were identified as *C. jejuni* by a complex of phenotypic traits.

Index	QMAFanM	BGKP	Campylobacter, including:			
			Microbiological method	By PCR for genes		
				16s rRNA for *C. jejuni* species	*cdtB*	For the genus *Campylobacter*
Detection frequency						
abs.	60	56	5	37	10	37
%%	100	93	8,3	61,6	16,6	61,6
Number, lg КОЕ/см^3				Value Ct		
M±m	5,2 ± 0,2	2,9 ± 0,2	1,3 ± 0,3	23,7 ± 0,8	31,4 ± 0,7	nd
Me	5,11	3,0	nd	22,5	32,9	nd
Min.	1,39	1,0	nd	12,7	21	nd
Макс.	8,13	7,0	2,0	34,8	38,8	nd
75%	6,38	4,0	nd	28,15	34,82	nd
90%	7,63	5,0	nd	31,48	35,97	nd

Table 2: *Characteristics of microbial contamination of raw milk (n = 60).*

nd: No Data; Ct for negative control of isolation when testing the 16s rRNA sequence -> 30.0; gene cdtB -> 33.0.

When studying the microbial background of the studied samples of raw milk, a comparative analysis of their contamination with *Campylobacter* by RT-PCR was carried out. Most of the samples (over 60%) were positive for the presence of the 16s rRNA genomic sequence, while they were characterized by the highest values of total bacterial contamination and the amount of BHCP.

Based on the results of PCR with genus-specific primers for *Campylobacter* spp. the rate of detection of *Campylobacter* in contaminated milk samples was 7.4 times higher than that for microbiological testing. Apparently, the culture method did not allow detecting the presence of the pathogen against the background of massive contamination with extraneous microflora, while the number of uncultivated or non-viable forms detected by PCR reached high values. A rough assessment of the results in a quantitative format indicated detectable levels of 104-106.5 genomic equivalents/cm^3, which indicates the possible presence of viable *Campylobacter* cells in the test material.

The use of a multi-primer approach (simultaneous testing for the presence of 16s rRNA and the *C. jejuni* cdtB gene) reduced the number of positive cases of *Campylobacter* DNA detection to 16.6%, which suggests that the cdtB gene is more informative in identifying viable, including uncultured, cells with toxicity.

To experimentally confirm the results obtained, studies were carried out to identify *C. jejuni* in model systems using milk artificially contaminated with test strain No. 11168 with 10-fold decreasing concentrations of microbial suspension (from 107 to 10 cells/cm3). Sterile milk and samples of native milk-raw materials with a high level of QMAFAnM and abundant seeding of BGKP were used as test objects of contamination (Table 3).

Calculated concentration of *C. jejuni*, cells /cm^3	**Detection results of *C. jejuni*, lg cells/cm^3**			
	Microbiological method, n = 3	**by RT-PCR with primers (n = 3)**		
		16s rRNA for *C. jejuni* species	***cdtB***	***ciaB***
Sterile milk contaminated with *C. jejuni*				
10^7	6,53 ± 0,35	6,5 ÷ 7,0	5,1 ÷ 5,4	5,6 ÷ 5,9
10^6	5,23 ± 0,14	6,0 ÷ 6,5	4,2 ÷ 4,6	4,5 ÷ 4,8
10^5	4,11 ± 0,27	5,0 ÷ 5,5	3,3 ÷ 3,8	3,0 ÷ 3,6
10^4	2,25 ± 0,56	4,0 ÷ 4,5	3,0 ÷ 3,5	3,3 ÷ 3,7
10^3	n/o	2,0 ÷ 2,5	n/o	n/o
10^2	n/o	0,5 ÷ 1,0	n/o	n/o
10^1	n/o	n/o	n/o	n/o
Control (no infection)	n/o	n/o	n/o	n/o
Bulk raw milk contaminated with *C. jejuni*				
10^7	5,23 ± 0,19	6,2 ÷ 6,7	5,0 ÷ 5,5	4,8 ÷ 4,9
10^6	3,94 ± 0,58	5,0 ÷ 5,4	4,0 ÷ 4,4	4,3 ÷ 4,5
10^5	3,76 ± 0,31	4,7 ÷ 4,9	3,0 ÷ 3,3	3,5 ÷ 3,8
10^4	1,95 ± 0,47	3,6 ÷ 4,1	2,2 ÷ 2,7	3,0 ÷ 3,2
10^3	n/o	1,5 ÷ 2,2	n/o	n/o
10^2	n/o	0,3 ÷ 0,8	n/o	n/o
10^1	n/o	n/o	n/o	n/o
The control	n/o	n/o	n/o	n/o
"÷" - detection range "N/a" - not found				

Table 3: *Detection of C. jejuni in experimental milk contamination.*

When contaminated with high doses (10^6 cells/cm^3) of the *C. jejuni* test strain, it was found that the detection of living (colony-forming) cells correlates to a greater extent with the results of PCR analysis with primers for the cdtB and ciaB genes, while the genomic sequence of 16s rRNA allows the detection of the presence of not only viable cells, but, possibly, inactivated or uncultivated forms of bacteria. At low concentrations of the inoculated pathogen (102103 cells/cm^3), positive PCR results were recorded only when tested with genus-specific

16s rRNA primers, while the culture method did not reveal *C. jejuni* at these levels of contamination. The sample opening in contaminated raw milk was almost an order of magnitude lower compared to contaminated sterile milk when tested by all methods, which may be due to the bacteriostatic properties of milk (due to the presence of the lactoperoxidase system, lysozyme, lactoferrin, immune antibodies, macrophages, etc.) and various forms of interpopulation interaction of representatives of extraneous microflora.

Thus, taking into account the need to identify unculturable *Campylobacter* forms in mixed microbial associations, the possibility of using the PCR method was shown, which avoids the main difficulty associated with testing such bacteria, especially those in a metabolically inactive state.

Conclusion

Using cultural and molecular genetic methods of direct detection based on PCR analysis, the nature of microbial contamination of raw cow's milk and the frequency of detection of thermophilic bacteria of the genus *Campylobacter* were studied.

It is shown that microbiological methods of inoculation at low levels of contamination of Campylobacteriosis pathogens do not ensure their reliable detection due to massive contamination of raw materials by extraneous microflora, which affects the test results. Positive results for *Campylobacter* spp. were obtained only in 8.3% of cases, while the use of multi-primer PCR analysis with the cdtB and ciaB genes makes it possible to double the detection of *C. jejuni* in raw milk samples.

The possibility of using PCR as the most adequate method was shown, which allows one to bypass the main difficulty associated with testing difficult-to-cultivate pathogens in a metabolically inactive state, providing the possibility of replacing bacterial reproduction by amplification of specific DNA fragments.

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Conflict of Interests

The authors declare no conflicts of interest.

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