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DETECTION OF *CAMPYLOBACTER* SPP. WITH REAL-TIME POLYMERASE CHAIN REACTION

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SUMMARY

Bacteria of *Campylobacter* genus are ones of the main zoonotic pathogens causing human and animal diseases. Campylobacter organisms are microaerophiles and, therefore, require low oxygen concentration (3–5%) and high carbon dioxide concentration (3–10%) for their growth. They use amino acids rather than carbons as a source of energy. Classical bacteriological methods for *Campylobacter* spp. detection are not always successful due to difficulties in creating optimal conditions for their growth. Therewith, development and implementation of molecular methods for *Campylobacter* detection and identification are of current importance. Assay for qualitative *Campylobacter* spp. detection with real-time polymerase chain reaction using CFX-96 thermocycler was optimized. Highly specific segment of 16S rRNA gene allowing identification of 6 *Campylobacter* species: *C. jejuni*, *C. coli*, *C. lari*, *C. upsaliensis*, *C. helveticus* и *C. hyointestinalis* was selected as an amplification target. Optimal magnesium ion concentration (2.5 мМ) and primer annealing temperature (58 °C) were determined. Eighteen reference strains of various bacteria were tested. Only tests of *Campylobacter* genus strains gave positive results. The method sensitivity was 40 target molecules. The said method was used for testing 76 samples of raw materials of animal origin. *Campylobacter* spp. genome was detected in 18 samples. Obtained results showed that the optimized variant of real-time polymerase chain reaction based on 16S rRNA gene amplification was a specific, sensitive, rapid, reproducible and accurate method for qualitative detection of *Campylobacter* spp. in samples of raw animal materials.

Key words: *Campylobacter* spp., real-time polymerase chain reaction, raw animal materials.

INTRODUCTION

Bacteria of *Campylobacter* genus are agents of infectious diseases that manifest themselves as abortions, temporal infertility, retention of placenta, vaginitis, metritis, unviable young animals in mammals as well as decreased gain weights in broilers and decreased egg production in hens and chick mortality in chickens. Moreover, these bacteria are a major cause of gastroenteritis in humans [9, 15, 19].

As at 2014, *Campylobacter* genus included 26 species and 9 subspecies [12]. Approximately a half of them is known pathogens of mammals but human diseases are mainly associated with *C. jejuni* and *C. coli*. There is evidence that other *Campylobacter* spp., such as *C. concisus*, *C. upsaliensis*, *C. hyointestinalis*, *C. fetus*, *C. curvas* and *C. lari* can also cause diseases in humans [8, 21]. Campylobacteriosis incidence is gradually increasing and currently *Campylobacter* is considered to be a major cause of bacterial gastroenteritis worldwide [5, 14, 25]. About 90% diarrheic diseases are considered to be caused by *Campylobacter* spp. [23]. Annually, 9.2 mln campylobacteriosis cases are reported in the European Union member states [10] and 1 mln campylobacteriosis cases are reported in the USA [13]. In Australia 22,564 cases associated with *Campylobacter* spp. infection were reported in 2015 [26].

Methods of laboratory campylobacteriosis diagnosis and in-process bacteriological control of animal products are based on the isolation of pure culture of the agent from animal biological materials by inoculations in blood and Erythrit agar with ferrum-sulfite-piruvate additives. All stages of *Campylobacter* spp.-inoculated media incubation are performed in microaerophilic atmosphere. *Campylobacter* organisms are cultivated at different temperatures for species differentiation [1, 2].

Routine *Campylobacter* spp. detection with the method including selective enrichment for inhibiting growth of competing microflora followed by biochemical

identification is mainly aimed at *C. jejuni* and *C. coli* detection. Other pathogens of *Campylobacter* spp. require additional conditions for their cultivation. For example, *C. concisus* is a slow-growing organism that requires hydrogen-enriched atmosphere. It is impossible to isolate target organism when the optimal growth conditions are not met [16]. Insufficient sensitivity of the cultivation methods, slow bacterium growth rate and incorrect identification with standard phenotypic methods could lead to false-negative results. Consequently, alternative rapid sensitive *Campylobacter* detection methods including real-time polymerase chain reaction (rtPCR) are required [18, 20, 22].

Accelerated methods significantly reduce test time (by 24–48 hours). Being highly specific, they enable reliable *Campylobacter* detection in tested materials [11, 27].

The study was aimed at optimization of the method for *Campylobacter* spp. genome detection in samples of animal products and raw animal materials with rtPCR.

MATERIALS AND METHODS

DNA extraction. Bacterial DNA was extracted with "Sorb-GMO-A" kit (OOO "Sintol", Russia) in accordance with the manufacturer's instruction.

Oligonucleotides. Primers and probes coding for 16S rRNA gene sequence for *Campylobacter* spp. identification used in the study and previously described by M. Lund et al. [8], were synthesized by the OOO "Sintol" (Russia). Oligonucleotide primary structures are presented in Table 1.

rtPCR conditions. rtPCR reagent kit produced by the OOO "Sintol" (Russia) was used. The reaction mixture was prepared from the following components as per sample (25 µl): 5 µl of DNA; 10× PCR-buffer; 2.5 µl of 2.5 мМ dNTP; 2.5 µl of 25 мМ $MgCl_2$; 0.3 pM forward primer and 0.3 pM reverse primer; 0.15 pM probe; 2.5 U of SynTaq DNA-polymerase.

DNA extracted from reference *C. jejuni* strain suspension (ATCC 29428) with optical density of 0.5 McFarland units (i.e. 1×10^8 CFU/cm^3) was used as a positive control. The suspension was prepared using 0.9% NaCl solution. Double-distilled water was used as a negative control.

Real-time amplification was carried out using CFX-96 thermocycler (Bio-Rad, USA) under the following conditions: mixture heating at 50 °C for 2 minutes; enzyme activation at 95 °C for 10 minutes; 45 cycles – at 95 °C for 15 s, at 58 °C for 1 min. The results were interpreted based on cycle threshold (Ct) value. The sample was considered positive when Ct ≤ 40.

Analytical sensitivity of rtPCR test system was determined using series of 10-fold dilutions of bacterial DNA extracted from reference *C. jejuni* strain suspension (ATCC 33291) with optical density of 1 McFarland unit that was equivalent to 3×10^8 CFU/cm^3. The suspension was prepared using 0.9% NaCl solution. Microorganism concentration was confirmed by titration onto solid nutrient medium, Columbia agar (HiMedia, India) supplemented with 5% defibrinated ram blood. Concentration of the extracted DNA was determined with Implen NanoPhotometer P-Class P-360 spectrophotometer (Implen, Germany). DNA dilutions were prepared using 100 µl of TE buffer.

Samples. Seventy-six samples of raw animal materials submitted for testing to the FGBI "ARRIAH" in 2017–2018 were used. The samples were classified into three groups: poultry – 13 samples; chicken meat preparations – 25 samples; raw cow milk – 33 samples, poultry blood – 4 samples; litter swabs – 1 sample.

Table 1
Nucleotide sequences of primers and probe for *Campylobacter* spp. genome segment detection

Description		Sequence (5'–3')
camp2	Forward:	CACGTGCTACAATGGCATAT
	Reverse:	GGCTTCATGCTCTCGAGTT
	Probe:	FAM-CAGAGAACAATCCGAACTGGGACA-RTQ1

Preparation of samples for testing. A sample weight (10 g or 10 cm^3) prepared for testing was added to 90 cm^3 of medium for *Campylobacter* initial enrichment (Bolton nutrient broth, produced by the FBIS "State Research Centre for Applied Microbiology and Biotechnology", Obolensk). Inoculated medium was incubated in anaerobic atmosphere at 37 °C for 4–6 hours, and then at 41.5 °C for (44 ± 4) hours. After initial enrichment completion, the inoculated medium was mixed and 250 µl of the suspension was taken from its middle part and transferred to 1.5 ml Eppendorf polypropylene tubes, and then tested with rtPCR.

The following reference strains obtained from the American Type Culture Collection (ATCC) were used for testing primers for their specificity: *Campylobacter jejuni* ATCC 33291; *Campylobacter coli* ATCC 43478; *Campylobacter lary* ATCC 35221; *Listeria monocytogenes* ATCC 19115; *Listeria innocua* ATCC 33090; *Listeria ivanovii* ATCC 19119; *Bacillus subtilis* ATCC 6633; *Bacillus cereus* ATCC 11778; *Enterococcus faecalis* ATCC 19433; *Rodococcus equi* ATCC 6939; *Pseudomonas aeruginosa* ATCC 9027; *Staphylococcus aureus* ATCC 6538 P; *Salmonella typhimurium* ATCC 14028; *Escherichia coli* ATCC 25922; *Shigella flexneri* ATCC 12022; *Shigella sonnei* ATCC 11060; *Proteus mirabilis* ATCC 29906; *Yersinia enterocolitica* ATCC 9610.

The non-target reference strains were grown in Columbian agar (HiMedia, India) and incubated at 37 °C for 24 hours. Reference *Campylobacter* strains were grown in Columbian agar (HiMedia, India) supplemented with 5% defibrinated ram blood and incubated at 37 °C for 24 hours in microaerobic atmosphere (CO_2 – 10%, O_2 – 5%, N_2 – 85%) according to the Methodical Guidelines (MG) 4.2.2321-08. Kampilogas gas generation bags (INKO, Russia) were used for creating these conditions.

Reference strains suspensions (density – 0.5 McFarland units) were used for tests with rtPCR. Density was measured with densitometer (BioMerieux, France) and confirmed by counting viable bacteria with method of 10-fold culture dilutions in plates containing Columbian agar (HiMedia, India) supplemented with 5% defibrinated ram blood for *Campylobacter* organisms.

Statistical data processing. Three replicate tests using 10-fold dilutions of genomic DNA were carried out for statistical processing of obtained results and linear regression curve plotting. Amplification effectiveness was calculated according to the following formula: $E = (10^{slope} - 1)\times100\%$, where 10^{slope} – slope coefficient. rtPCR results were analyzed with v3.1 system software (CFX Manager Software).

RESULTS AND DISCUSSION

Sequences coding for 16S rRNA gene segment were selected based on the results of literature data

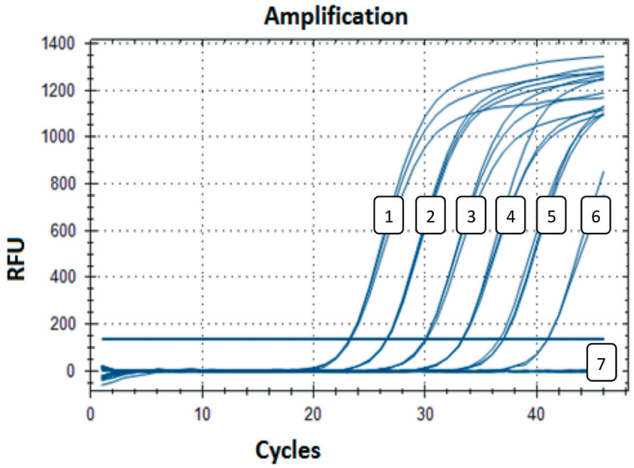

Fig. 1. Graph of fluorescent signal accumulation in case of Campylobacter spp. genome detection with rtPCR (n = 3)

Bacterial cell concentrations:
1 – 4×10⁵; 2 – 4×10⁴; 3 – 4×10³; 4 – 4×10²; 5 – 40; 6 – 4; 7 – 0.4.

analysis (Table 1) [8]. The said primers identify four species of thermophilic *Campylobacter* organisms: *C. jejuni*, *C. coli*, *C. lari*, *C. upsaliensis*, that mainly occur in poultry and are pathogenic for humans [4, 17]. Moreover, these primers are used for *C. helveticus* and *C. hyointestinalis* identification, the first one mainly occurs in cats and dogs [6], the second one – in pigs [7].

Optimization of rtPCR for Campylobacter spp. detection included determination of primer annealing temperature and magnesium ion concentration for which the highest responsive fluorescent signal intensity was observed with high specificity. Optimal annealing temperature was determined based on the primer structure [3]. Based on calculations made according to the formula: Tm (°C) = 2×(A+T) + 4×(G+C), where Tm – annealing temperature; A, T, C, G – nucleotide bases, annealing temperature for forward and reverse primers was 58 °C, probe annealing temperature was 72 °C. rtPCRs were carried out at calculated primer annealing temperature using a gradient of ±10 °C. The best results were obtained when the temperature was 58 °C.

PCR mixtures with concentrations of 2–6 мМ and gradient of 0.5 мМ were used for determination magnesium ion concentrations. The concentration of 2.5 мМ was selected based on the test results.

Optimal conditions under which Ct value was the least at minimum primer and probe concentrations are described in Materials and Methods.

Table 2
Ct value when *Campylobacter* spp. genome was detected with rtPCR (*n* = 3)

No.	Bacterial cell concentration	Final RFU	Ct value	Mean Ct value	Standard deviation, ±SD	Result
1	4×10⁵	1,163	23.34	23.26	0.072	(+) positive
2		1,337	23.24			(+) positive
3		1,269	23.20			(+) positive
4	4×10⁴	1,234	26.62	26.57	0.051	(+) positive
5		1,262	26.59			(+) positive
6		1,286	26.52			(+) positive
7	4×10³	1,244	30.03	30.07	0.058	(+) positive
8		1,171	30,05			(+) positive
9		1,096	30.14			(+) positive
10	4×10²	1,093	33.40	33.35	0.044	(+) positive
11		1,065	33.32			(+) positive
12		1,216	33.33			(+) positive
13	40	1,011	37.02	36.91	0.226	(+) positive
14		980	37.06			(+) positive
15		1,004	36.65			(+) positive
16	4	-0.668	n/d	41.02	0.035	(–) negative
17		549	41.04			(–) negative
18		501	40.99			(–) negative
19	0.4	-0.666	n/d	-	-	(–) negative
20		-1.05	n/d			(–) negative
21		-3.75	n/d			(–) negative

n/d – not detected.

Analytical sensitivity. DNA extracted from control *C. jejuni* strain suspension (ATCC 33291) with optical density of 1 McFarland unit that was equivalent to 3×10^8 CFU/cm^3 was used for testing the method for its analytical sensitivity. DNA concentration was determined with IMPLENP-class P-360 nanophotometer (Germany) and was 12.4 ng/µl.

Considering the middle size of genomes of different tested microorganisms, one ng of genomic DNA corresponds to at least 3×10^5 cells [24]. Thus, DNA concentration of 12.4 ng/µl corresponds to approximately 4×10^6 bacterial cells in 1 µl. Extracted DNA was diluted with TE buffer using gradient of 10 and amplification reaction was carried out in triplicate using the following bacterial cell concentrations in the samples: 4×10^5; 4×10^4; 4×10^3; 4×10^2; 40; 4; 0.4 (Fig. 1).

Mean Ct value and standard deviation (±SD) were calculated for each dilution (3 replicates) (Table 2).

Obtained results showed that 16S rRNA gene fragment was amplified in the first five dilutions.

Three replicate tests were performed to assess the method performance and Ct values were derived to plot regression curve (Fig. 2). Quantities of target DNA were found to linearly correlate to Ct values at correlation coefficient of 0.9986. Slope angle value derived by plotting linear regression curve was used to determine amplification effectiveness and was E = 92.26%.

Regression analysis indicates linearity of obtained results. Amplification effectiveness (E = 92.26%) and correlation coefficient (R^2 = 0.9986) show that the optimized assay enables highly accurate *Campylobacter* spp. detection.

Determination of specificity. DNA isolated from 18 bacterium species including three target (*C. jejuni*, *C. coli*, *C. lari*) and 15 non-target strains were tested for its cross reactivity to determine rtPCR assay specificity (Fig. 3).

FAM fluorescence values enabled unambiguous identification of all strains belonging to *Campylobacter* genus whereas no fluorescence was observed for non-target strains. The results proved that the optimized method was highly specific. Equal effectiveness of all reactions specific for *Campylobacter* species is crucial for successful method application. Analysis of fluorescence profiles derived during specificity tests showed that Ct values were similar and did not depend on particular *Campylobacter* species (mean Ct value was 21.39 ± 0.74) (Fig. 3).

Thus, proposed rtPCR assay enables highly specific identification of target strains of *Campylobacter* genus.

Detection of Campylobacter spp. genome in raw animal materials. Seventy-six (76) samples of raw animal materials were tested with the optimized rtPCR assay (Table 3).

Campylobacter spp. genome was detected in 18 samples. The highest number of positive samples (44.0%) were detected in chicken meat preparations. *Campylobacter* genome was also detected in poultry carcasses (23.1%), poultry blood (2.0%), raw cow milk (9.1%).

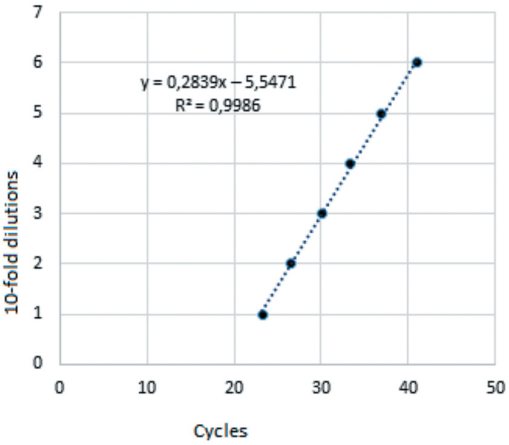

Fig. 2. Linear regression graphs of Ct when 10-fold dilutions of Campylobacter spp. DNA were tested with rtPCR

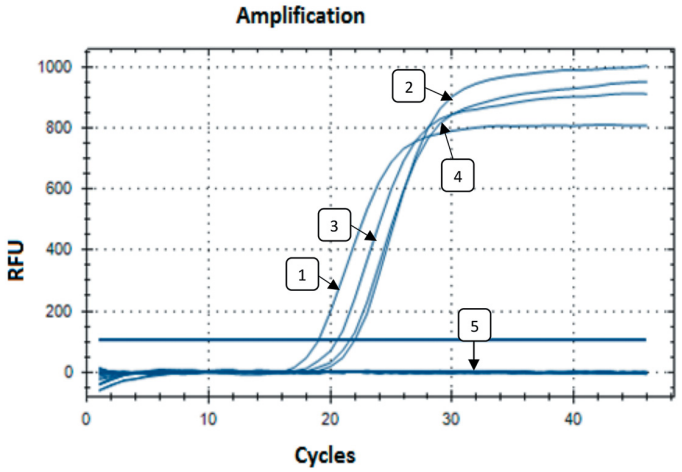

Fig.3. Specificity of rtPCR for Campylobacter spp. genome identification

1 – positive control;
2 – Campylobacter jejuni ATCC 33291;
3 – Campylobacter coli ATCC 43478;
4 – Campylobacter lary ATCC 35221;
5 – non-target strains.

Table 3
***Campylobacter* spp. genome detection in raw animal material samples with rtPCR assay**

Group No.	Sample name	Number of samples		
		total	positives	%
1	Chicken meat preparations	25	11	44.0
2	Poultry	13	3	23.1
3	Raw cow milk	33	3	9.1
4	Poultry blood	4	1	25.0
5	Litter swabs	1	0	0
	Total	76	18	23.7

CONCLUSION

The assay enabling detection of *Campylobacter* spp. genome in case of presence of approximately 40 target molecules was optimized based on the performed tests. The following rtPCR conditions were optimized: optimal magnesium concentration (2.5 мМ) and primer annealing temperature (58 °C) were selected. Selected primers were found to be highly specific and give no false positive reactions. The assay was shown to enable accurate detection of *Campylobacter* spp. (R^2 value > 0.99) with PCR effectiveness of 92.26%.

The assay was approved for use under laboratory conditions upon testing 76 samples of raw animal materials (chicken products, raw cow milk, chicken biomaterials).

The test results suggest that the method is highly specific, sensitive and is able to detect *Campylobacter* spp. in tested samples.

Thus, proposed assay can be used as a rapid method in addition to classical methods applied for routine analysis at microbiological laboratories. Moreover, the said method can be used for assessment of *Campylobacter* spp. occurrence in food industry as well as in raw and processed products of animal origin.

Conflict of interest. The authors declare no conflict of interest.

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