**Clostridium spp. detection in food samples using 16S rDNA-based PCR method**

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**Abstract.** Raw foods of animal and plant origin are the most likely to be contaminated with either spores or vegetative cells of Clostridium. Molecular detection of the Clostridium spp. by polymerase chain reaction (PCR) is one useful method for detecting Clostridium spp. in some samples such as food raw materials. Clostridium detection was performed using 16S rDNA-based PCR method with Clos 58 - f and Clos 780 - r primers. A study of the specificity of the primers used revealed a reaction to Salmonella and Proteus. Therefore, the scope of PCR with these primers can be food products that have undergone high-temperature processing, for example, sterilized meat or meat and vegetable preserves, as well as ingredients after inactivation of vegetative forms of microorganisms.

**1. Introduction**

Raw foods of animal and plant origin are the most likely to be contaminated with either spores or vegetative cells of Clostridium. Also, food can get contaminated in the field, during processing, or during other stages in the food production chain, including through cross-contamination. The main factor of Clostridium contamination of animal origin foods is due to contamination during slaughtering and processing. Clostridium contamination of plant is often due to soil pollution, poor-quality washing and cleaning. In addition, non-compliance with the rules for the separate storage of products may cause raw materials re-contamination [1].

According to the Netherlands National Institute of Public Health and the Environment report, the contamination of C. perfringens food leads to 160 thousand outbreaks of foodborne infections each year. The main sources of the disease are meat-containing soups and stews, herbs and spices [2]. Food poisoning caused by Clostridium is often associated with the consumption of meat products (boiled meat stored at room temperature, cold meat snacks; pies with liver), canned products, spices. There are no exactly statistics on human diseases caused by sterilized canned meat products consumption in the Russian Federation. At the same time, botulinum toxin is one of the most toxic natural substances in the world. It can accumulate in sterilized canned food. After commercial sterilization, the residual microflora represented by clostridia can be in canned food. In Russia there are following methods for Clostridium detection: GOST 29185-2014 (ISO 15213:2003) Microbiology of food and animal feeding stuffs. Methods for detection and the enumeration of sulftite-reducing bacteria growing under anaerobic conditions, GOST 29185-2014 (ISO 15213:2003) Microbiology of food and animal feeding stuffs. Methods for detection and the enumeration of sulfite-reducing bacteria growing under anaerobic conditions and GOST 30425-97 Canned foods. Method for determination of commercial...
sterility. The basis of these standards is the bacteriological culture method. The method includes determining the cultural and morphological characteristics of growth, sulfite-reducing activity, anaerobic growth, the absence of catalase reduces. Molecular detection of the Clostridium spp. by polymerase chain reaction (PCR) is one useful method for detecting Clostridium spp. in some samples such as food raw materials [3, 4]. Rapid detection methods are important in food industry, as they are able to detect the microorganisms in raw and processed foods immediately [5]. Rapid methods are also sensitive enough to detect pathogens that present in low numbers in the food [6, 7]. It was possible to detect 4–8 spores per ml for different Clostridium species, without the need for enrichment of the samples [8]. This is important for detection of clostridium-contaminated products immediately. There are studies demonstrated the use of molecular techniques for determining bacterial species (including Clostridium) and monitoring pathogens in the chick gastrointestinal tract [9, 10]. Microflora was determined using 16S ribosomal DNA (rDNA) targeted probes from bacterial DNA [11]. This approach can be used for determining bacterial species (including Clostridium) in food samples.

2. Materials and methods

2.1. Samples
Strains: Clostridium perfringens ATCC 13124, Clostridium sporogenes ATCC™ 19404, Proteus mirabilis ATCC 35659, Salmonella enterica subsp.enterica serovar Enteritidis ATCC™ 13076, Salmonella enterica subsp.enterica serovar Gallinarum ATCC® 13036™, Salmonella enterica subsp.enterica serovar Typhimurium ATCC 14028.

2.2. Microbiological method
For DNA extraction, museum strains were cultured on liquid culture media. DRCM broth (Merck, Germany) was used as a liquid culture medium for the Clostridium growth. TSB (Merck, Germany) was used as a liquid culture medium for the Salmonella and Proteus growth. DNA extraction was performed after 24 h at 37 ° C.

2.3. DNA extraction
Next, 1 mL of each sample suspension was centrifuged at 3286 g for 5 min at microcentrifuge MiniSpin (Eppendorf, Hamburg, Germany). DNA extraction was performed on MagNA Pure LC 2.0 Instrument (Roche, Switzerland) using MagNa Pure LC DNA Isolation Kit III (Roche, Switzerland).

2.4. PCR
Alignment of primer sequences was performed using Primer-BLAST programme and Oligo Analyzer 3.1.

Real-time PCR was performed on «ANK 32» (OOO "Syntol", Russia). PCR primers used in the study are presented in the table.1.

Table 1. PCR primers used in the study.

| Bacterial group | Primers | Sequence (5′-3′) | Length (bp) | References |
|-----------------|---------|------------------|-------------|------------|
| Clostridium     | Clos58-f| AAAGGAAGATTAATACGCATAA | 722         | E.Amit-Romach et. al., 2004; Seyed Ziaeddin Mirhosseini et. al., 2010 |
|                 | Clos780-r| ATCTTGCGACCCTAATCCCCC |             |            |
3. Results and discussion

3.1 Table 1 shows the results of the assessment of the forward-primer Clos58-f specificity in relation to non-target species of microorganisms.

The sequence coincides not only within the genus Clostridium, but also within the family Enterobacteriaceae. For example, the probability of a forward primer sequence coinciding with Salmonella enterica subsp. enterica serovar Enteritidis was 65.5% versus 100% match with Clostridium diolis. Similar results were obtained when evaluating the specificity of the reverse primer Clos780-r. The probability of a reverse-primer sequence coinciding with Salmonella enterica subsp. enterica strain 12-0523 chromosome, complete genome was 75.3% versus 100% with Clostridium diolis.

**Table 2. Alignment of forward-primer sequence.**

| Organism                        | Blast Name         | Score | Number of Hits | Description                                      |
|---------------------------------|--------------------|-------|----------------|--------------------------------------------------|
| Bacteria                        | bacteria           | 973   |                |                                                  |
| Clostridium                     | firmicutes         | 174   |                |                                                  |
| Clostridium diolis              | firmicutes         | 46.1  | 1              | Clostridium diolis hits                          |
| Clostridium butyricum           | firmicutes         | 46.1  | 18             | Clostridium butyricum hits                       |
| Clostridium chauvoel            | firmicutes         | 46.1  | 3              | Clostridium chauvoel hits                        |
| Clostridium beijerinckii NRRL B-593 | firmicutes       | 46.1  | 1              | Clostridium beijerinckii NRRL B-593 hits          |
| Clostridium perfringens         | firmicutes         | 46.1  | 15             | Clostridium perfringens hits                     |
| Clostridium botulinum           | firmicutes         | 46.1  | 40             | Clostridium botulinum hits                       |
| Clostridium acetobutyllicum     | firmicutes         | 46.1  | 2              | Clostridium acetobutyllicum hits                  |
| Clostridium beijerinckii        | firmicutes         | 46.1  | 5              | Clostridium beijerinckii hits                     |
| Clostridium sp. MF28            | firmicutes         | 46.1  | 1              | Clostridium sp. MF28 hits                        |
| Clostridium chauvoei JF 4335    | firmicutes         | 46.1  | 1              | Clostridium chauvoei JF 4335 hits                 |
| Clostridium saccharobutylicum   | firmicutes         | 46.1  | 7              | Clostridium saccharobutylicum hits                |
| Clostridium saccharoperbutylacetonicum | firmicutes       | 46.1  | 1              | Clostridium saccharoperbutylacetonicum hits      |
| Clostridium tyrobutyricum       | firmicutes         | 46.1  | 2              | Clostridium tyrobutyricum hits                    |
| Clostridium sporogenes          | firmicutes         | 46.1  | 3              | Clostridium sporogenes hits                       |
| Clostridium scatologenes        | firmicutes         | 46.1  | 1              | Clostridium scatologenes hits                     |
| Clostridium autoethanogenenum   | firmicutes         | 38.2  | 2              | Clostridium autoethanogenenum DSM 10061 hits      |
| DSM 10061                       |                    |       |                |                                                  |
| Clostridium carboxidivorans P7  | firmicutes         | 38.2  | 1              | Clostridium carboxidivorans P7 hits               |
| Clostridium baratii str Sullivan| firmicutes         | 38.2  | 1              | Clostridium baratii str Sullivan hits             |
| Clostridium botulinum 202F      | firmicutes         | 38.2  | 1              | Clostridium botulinum 202F hits                   |
| Clostridium tetani 12124569     | firmicutes         | 38.2  | 1              | Clostridium tetani 12124569 hits                  |
| Clostridium pasteurianum BC1    | firmicutes         | 38.2  | 1              | Clostridium pasteurianum BC1 hits                 |
| Clostridium botulinum BKT015925 | firmicutes         | 38.2  | 1              | Clostridium botulinum BKT015925 hits              |
| Clostridium ljungdahii DSMb13528| firmicutes         | 38.2  | 1              | Clostridium ljungdahii DSMb13528 hits             |
| Clostridium botulinum E3 str Alaska E43 | firmicutes | 38.2  | 1              | Clostridium botulinum E3 str Alaska E43 hits      |
| Clostridium novyi NT            | firmicutes         | 38.2  | 1              | Clostridium novyi NT hits                         |
Testing primers on museum strains.

Logarithmic growth of Cl. perfringens and Cl. sporogenes passes on 12-13 cycles, and the growth of amplification curves of microorganisms of the genus Salmonella and Proteus on 22-23 cycles (figure 1). The melting point of the resulting amplicons from Clostridium and Salmonella was identical and was 88.5° C the melting point of the amplicons from protea was 87 ° C (figure 2).

![Amplification Curves](image)

Figure 1. Amplified curves: 1 - Clostridium perfringens ATCC 13124; 2 - Clostridium sporogenes ATCC™ 19404; 3 - Salmonella enterica subsp.enterica serovar Gallinarum ATCC® 13036; 4 - Salmonella enterica subsp.enterica serovar Typhimurium ATCC 14028; 5 - Salmonella enterica subsp.enterica serovar Enteritidis ATCC™ 13076; 6 - Proteus mirabilis ATCC 35659; 7 – negative control (water).
Figure 2. Melted curves: 1 - Clostridium perfringens ATCC 13124; 2 - Clostridium sporogenes ATCC™ 19404; 3 - Salmonella enterica subsp. enterica serovar Gallinarum ATCC® 13036; 4 - Salmonella enterica subsp. enterica serovar Typhimurium ATCC 14028; 5 - Salmonella enterica subsp. enterica serovar Enteritidis ATCC™ 13076; 6 - Proteus mirabilis ATCC 35659; 7 – negative control (water).

The data obtained show that primers Clos58-f and Clos780-r are annealed not only on the DNA of target microorganisms of the genus Clostridium [1, 5], but also on the DNA of non-target microorganisms, for example, Salmonella.

4. Conclusion
Currently, microbiological methods of food analysis on clostridia are often time-consuming. In this study, PCR was used to allow identify the majority of sulfite-reducing clostridia. A study of the specificity of the primers used revealed a reaction to Salmonella and Proteus. Therefore, the scope of PCR with these primers can be food products that have undergone high-temperature processing, for example, sterilized meat or meat and vegetable preserves, as well as ingredients after inactivation of vegetative forms of microorganisms. Sterilization of canned foods provide for the death of vegetative microflora and allow the survival of spores of microorganisms, for example, the genus Clostridium. Using these primers will allow you to control microbiological risks in the manufacture of products using anaerobic packaging.

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