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Creation of a Real-Time Polymerase Chain Reaction (RTPCR) Test System for Express Diagnostics of *Clostridium septicum*, *Clostridium perfringens*, *Clostridium chauvoei*, *Clostridium novyi* in Products of Animal Origin and in Environmental Objects

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Abstract: Livestock breeding in the republic causes significant damage from dysentery of lambs, brodzot and enterotoxemia including emphysematous carbuncle affecting cattle. Do not avoid animals of all kinds of disease malignant edema, necrobacillosis, tetanus, botulism. The main reservoir and location of clostridia is the soil. It serves as a potential source of anaerobic infections in animals and poses a certain threat of outbreaks, depending on the natural and climatic characteristics of the formation of emergent situations for clostridiosis. Bacteria of the genus clostridium (Clostridium) are among the microorganisms that have universal distribution. They often cause severe diseases which can result in death.

INTRODUCTION

On the territory of Kazakhstan on clostridiosis, an important place is occupied by infectious enterotoxemia, bridzote, malignant edema, necrotic hepatitis, anaerobic dysentery of lambs, emphysematous carbuncle, etc. Clostridia often cause wound (gangrene and postpartum complications (endometritis, vaginitis, mastitis) as well as toxicoenteritis in humans, animals and birds[1, 2].

Food products (meat products, fruits and vegetables, milk and dairy products), for clostridiosis are products that create anaerobic conditions without preliminary heat treatment (canned goods, pickles, smoked products, dried foods, home-made sausages). The contact-home mechanism is realized through the wound path of infection when spores of clostridia of certain species fall on damaged skin[3].

The diagnosis of clostridiosis is based primarily on clinical data, since clostridia are often excreted from wound contents and without the development of clostridiosis[4, 5].

A modern approach to the study of clostridiosis is the use of molecular genetic methods of investigation. These methods are various modifications of the Polymerase Chain Reaction (PCR). Advantages of PCR are due to the practical simplicity and speed, sensitivity and specificity of this method. In this regard, it is extremely important to develop and actively introduce diagnostic methods in the work of veterinary laboratories based on the identification of unique fragments of the genome of the pathogen. The indicated methods of detection of infection by the causative agent. *C. perfringens*, *C. chauvoei*, *C. novyi*, *C. septicum* are promising. High sensitivity, specificity and relative simplicity of the method allows it to be used in the diagnosis.

MATERIALS AND METHODS

The following were analyzed in the studies: the 38 strains of the genus Clostridium (*C. septicum*, *C. perfringens*, *C. chauvoei*, *C. novyi*).

Isolation of DNA: To obtain cultures of Clostridium, pathogens were grown on specialized media. Isolation was performed using the commercial kit "PureLink® Genomic DNA Kits" produced in the USA. The procedure for DNA isolation was performed according to the instructions attached to the DNA extraction kit "PureLink".

The DNA concentration was determined using a Halo DNAmaster Dynamica spectrophotometer ("Dynamica GmbH", UK). Before introducing into the reaction mixture, the DNA isolated from the cultures was diluted to 10 ng mL^{-1}.

Selection of specific primers and fluorescently-labeled probes: The search for nucleotide sequences for the selection of specific primers was performed using GenBank NCBI online program (http://www.ncbi.nlm.nih.gov/GenBank). Alignment of nucleotide sequences was performed using the Vector NTI Advance program suite. The primers and probes were operative using Oligo 6.71.

PCR and analysis of results: The amplification was carried out using the QuantStudio 5 Real-TimePCRSystem thermal cycler ("AppliedBiosystems™," USA). The composition of the reaction mixture consisted of: 18 μL of a 1.25×PCR buffer, 0.24 μL of 25 mMdNTPs, 0.125 μL of each primer (100 μM), 0.14 μL probe (50 μM), 10 μL Taq polymerase solution, 5 μL of the DNA solution. As the reaction proceeds, a dependence of the fluorescence level on the cycle number is determined for each sample and a corresponding graph is plotted using the software attached to the amplifier. A threshold analysis method was used to determine Cq values. Primers and fluorescently-labeled probes were synthesized in ZAO "Sintol" (Moscow).

PCR was performed in accordance with the following amplification program: 95°C-4 min; 95°C-30 s, 58°C-30 s, 72°C-20 c×30 cycles; 72ºC-5 min.

Cloning positive controls: With the primers chosen earlier, amplification was carried out with obviously positive samples. Then, the amplified region was inserted into the plasmid T-vector (T-vector-has a T segment at the ends (thymine), the length of the vector was 3 kb).

The amplification was incubated with the polymerase, dNTA was added and placed for 45 min at 72°C, for the synthesis at the A-adenine ends. The resulting inserts were incubated with the T vector at 30°C for 30 min with addition of ligase and ligase buffer. The concentration of inserts was 2-4 times greater than the vector.

Preparation of cells: *E. coli* cell cultures with a ΔM13 mutation were used. From this culture, chemically competent substances were obtained under the influence of temperature (hit-shock, rapid heating). The frozen cell suspension was placed in 1.5 mL of the tube in a volume of 50+0.5-5 μL of the vector with the insert and left on ice for an hour, during which time the vectors were adsorbed on the cell surface. Then it was sharply heated in a prepared water bath (42°C) for 30 sec-1.5 min while in the cytoplasmic membranes pores were formed, through which the vectors penetrated.

The resulting suspension was transferred to an enriched nutrient medium and incubated for one hour with periodic shaking (to ensure aeration). They made a medium (1.5-2%) containing antibiotic (ampicillin) on LB. Incubated for 12-24 h, after incubation, only white colonies were selected (because only the vector with insertion was integrated in them).

Plasmid DNA was isolated by commercial kits and tested for its quality in a 0.7-1% agarose gel. At the same time, two bands (twisted plasmid and torn fragment) were visible in the upper part of the staircase and also in the lower part was seen bend-short sections of RNA. The molecular weight of the fragments was estimated using DNA markers with a molecular weight of 50 base pairs of GeneRuler. The results of electrophoresis were visualized on the gel-documenting system QUANTUM.

To establish the length of the plasmid and its purification, it was placed on restriction. The restriction site was in the inserted site. After this, the ring plasmid becomes linear and one band (3kb+amplified section) is visible on the foreface.

After that, the concentration of DNA was measured: As a result, a positive control sample was obtained. As a PC, a plasmid construct with an insert size of 170 bp was used for *Clostridium septicum*, 286 bp for *Clostridium perfringens*, 263 p for *Clostridium chauvoei* and 205 bp for *Clostridium novyi* flipped with inverted sequences homologous to the primer used to amplify the internal control sample. One specific fragment for each primer pair was used to clone positive controls.

RESULTS AND DISCUSSION

Search for specific markers in the genome of the microorganisms: To date, the decoding of the genome of bacteria of the genus Clostridium has not been completed. However, the genomes of the following clostridial species have been deciphered: *C. perfringens* str. 13, *C. botulinum E3* str. AlaskaE43, *C. difficile* 630 and *C. tetani*. All of the clastridial clostridia have a circular genome.

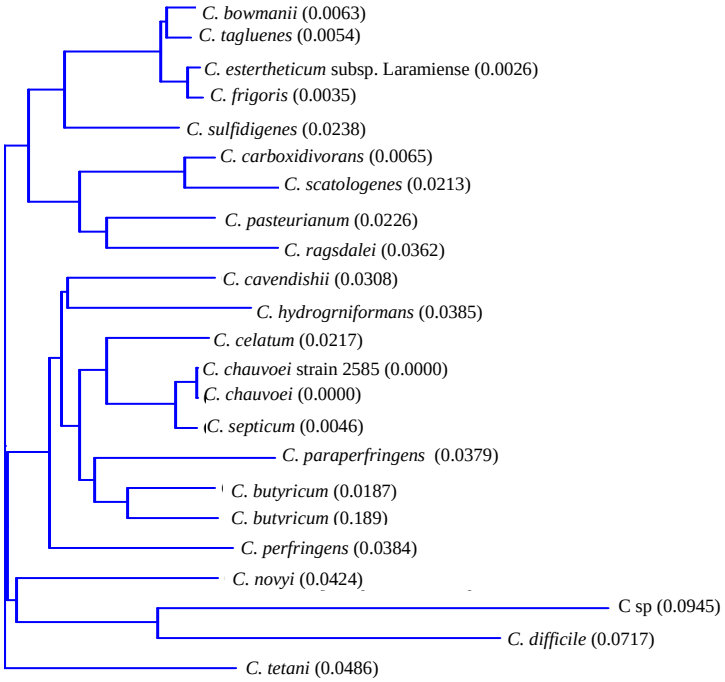

Fig. 1: Undeveloped phylogenetic tree, constructed using the vector NTI program complex, based on alignment of nucleotide sequences of microorganisms of the genus Clostridium (0.1-evolutionary distension)

In the genome of *C. perfringens* 3031430 pairs of nucleotide sequences with a GC content of 28%. There are 2,786 genes of which 2,660 are coding proteins. The genome of *C. tetani* contains 2799255 pairs of nucleotides with a GC content of 28%. There are 2,445 genes of which 2,373 are coding proteins. The genome of *C. difficle* has 3256683 bp, the GC content is 28%; 3017 genes of which 2876 are coding proteins. The genome of *C. botulinum* has 3659644 bp, the GC content is 27%; 3381 genes of which 3256 are coding proteins. Thus, the sequences of the bases of the genome of *C. chauvoei*, *C. novyi*, *C. septicum* can vary widely. The genome above these bacteria can contain from 2-4 million bp.

The target genes in the assay were: phospholipase C gene (plc), flagellin (fliC) gene, alpha toxin gene, gyrA, groEL, tpi, colA and glpK.

Selection of specific primers and fluorescently-labeled probes: By aligning the genes of GenBank nucleotides of these genes by the ClustalW algorithm and analyzing them in the Oligo 6.71 program, polymorphic and/or conservative loci were identified which in their parameters, were suitable for the selection of primers and probes. The next stage was the alignment of gene sequences with similar sequences of related organisms. To create primers and probes for *C. perfringens*, *C. chauvoei*, *C. novyi*, *C. septicum*, the following genes were selected in the NCBI database: the phospholipase C gene (plc), the flagellin gene (fliC), the toxin alpha gene, gyrA, groEL, tpi, colA and glpK. The next step, after searching for markers, was the selection of specific primers. It was necessary to establish which fungi are the closest phylogenetic relatives to the desired organisms. To this end, we built a phylogenetic tree for microorganisms of the genus Clostridium (Fig. 1).

After the construction of the trees, alignment was performed, using the nucleotide sequences of the closest species. Sequence alignment is a bioinformatic method based on the placement of two or more sequences of DNA, RNA or protein monomers under each other in such a way that it is easy to see similar sites in these sequences (Fig. 2).

For each of the above genes, a sequence was searched which included not only target organisms but also a group of closely related organisms to this species. Next, the alignment was performed using the "Vector NTI" program component-AlignX. Specific sites suitable for the creation of primers and probes have been found only of alpha toxin (*Clostridium septicum*), the phospholipase C (plc) gene (*Clostridium perfringens*, *Clostridium novyi*) and the flagellin gene (fliC) (*Clostridium chauvoei*) (Table 1).

When studying the results of equalization for the remaining selected genes, it was not possible to find unique, specific sites that would be suitable for the creation of primers. In some cases, no specific sites were

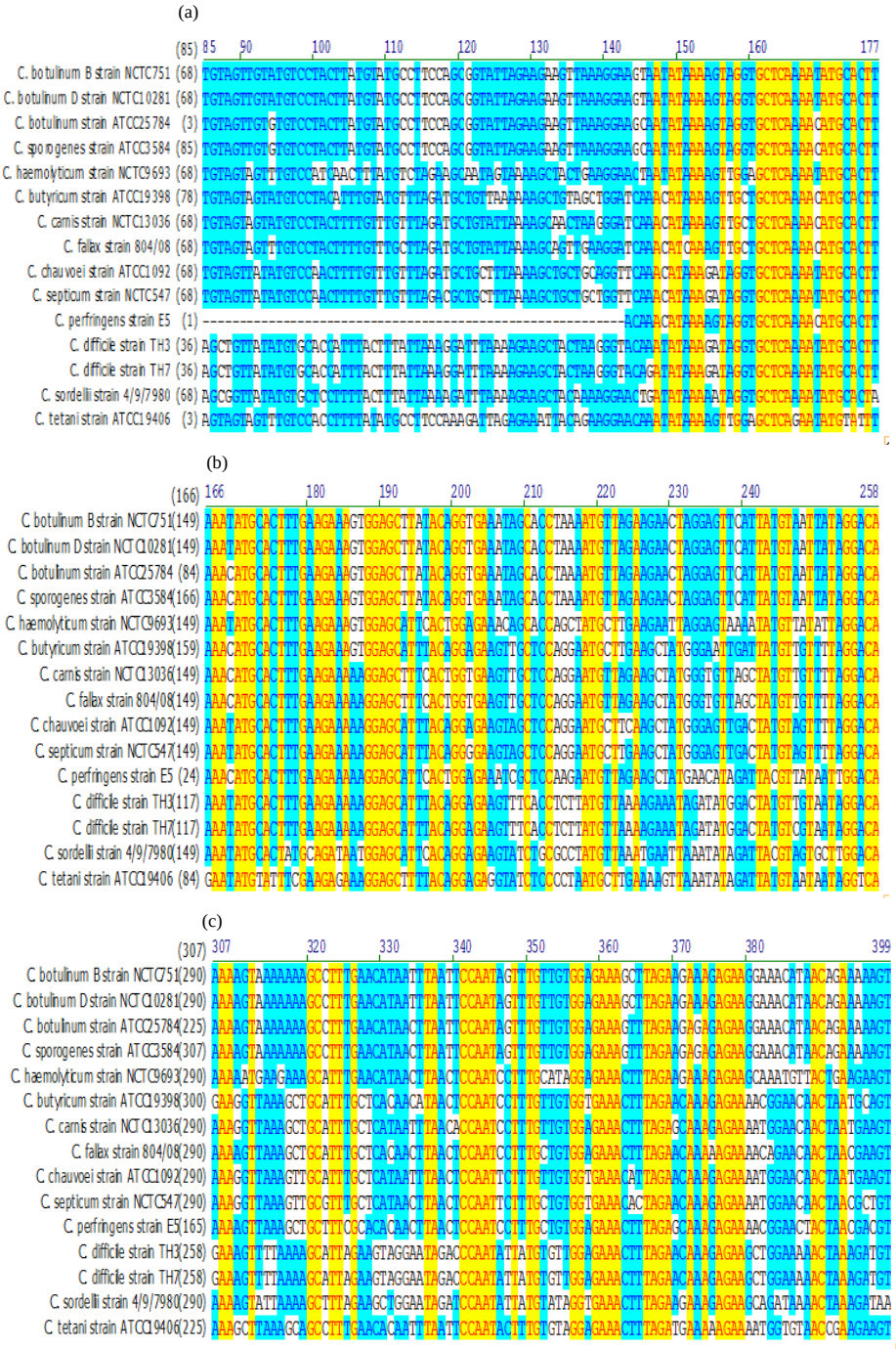

Fig. 2(a-c): Alignment of a fragment of the gene of microorganisms of the genus Clostridium (yellow areas indicate complete coincidence of nucleotides, blue-partial coincidence, green-50% coincidence, colorless areas-unique sequences)

found for the targeted organisms (yellow or blue coloration) in almost all the nucleotide sequences in other situations when the appropriate sites were found, unsatisfactory results of the "BLAST analysis" or the melting point were obtained was not suitable for PCR as well as oligonucleotides containing defective regions (so-called dimers, tails and loops) were eliminated.

In the future, for each stage of amplification, the temperature and time were selected experimentally. The

Table 1: List of selected oligonucleotides

View pathogen	Gene	Oligonucleotide	Length (bp)
Clostridium septicum	Alpha-toxin	Upper primer 5'-CGGCAGTAGTACCACATGTA-3'	21
		Lower primer 5'-TACATTTGCCCAATTTCTTA-3' (taagaaattgggcaaatgta)	20
		Probe 5'-(BHQ1)-GGGGATATGCAAA(FAMdT)CATAATAATGCTTCTT-3'	30
Clostridium perfringens	Phospholipasae C (plc)	Upper primer 5´-GCTAATGTTACTGCCGTTGATA-3'	22
		Lowerprimer 5'-CTGTTCCTTTTTGAGAGTTAGCT-3' (agctaactctcaaaaaggaacag)	23
		Probe 5'-(BHQ1)-AGTCCCAATCA(FAMdT)CCCAACTA GACTCATGC-3'(gcatgagtcatagttgggatgattgggact)	30
Clostridium chauvoei	Flagellin (fliC)	Upper primer 5'-AACGTAGCTGTAGATAGAACTGC-3'	23
		Lowerprimer 5´- CTTCCATCAGCTAAGTTACTATTT-3' (aaatagtaacttagctgatggaag)	24
		Probe 5´-(BHQ1)-TTAGATGGTGGT(FAMdT)TCAAAGGAGAATTCCAA-3'	30
Clostridium novyi	Phospholipasae C (plc)	Upperprimer 5'-GGAGCATCAAGTAAAGCGTA-3'	20
		Lowerprimer 5'-CATTCGGATCATAATCAGGA-3' (tcctgattatgatccgaatg)	20
		Probe5´-(BHQ1)-GAACTCATTCCA(FAMdT)GATTGTAACACAAGCAG-3'	30

temperature of the denaturation stage was chosen between 92-95°C, annealing between 58-66°C and the elongation between 70-75°C.

Based on the optimal time and temperature parameters chosen during the experiments for all the stages of amplification, the following mode was developed for PCR:

- Pre-denaturation-95°C-4 min
- Denaturation-95°C-30 s
- Annealing: 58°C-30 with 30 cycles
- Synthesis: 72°C-20 s
- Post-replication 72°C-5 min

After the time and temperature conditions of the amplification were determined, the content of the components in the reaction mixture was optimized and the PCR was perfected.

The concentration of MgCl2 largely determines the specificity and sensitivity of PCR, affects the primer annealing, the denaturation of the sample, in addition, $MgCl_2$ is necessary to maintain the activity of Taq polymerase. However, an excess of magnesium chloride can cause the production of a non-specific amplificate. In the case of a change in concentration, a qualitative or quantitative change in the resulting amplification occurs. To select the optimal concentration of magnesium chloride, reaction mixtures with the following concentration of MgCl2 were used: 1.5, 2, 2.5, 3, 3.5, 4 and 4.5 mM.

During the analysis of the results, it was noted that the amplification was equally effective at a broad Mg^{2+} ion concentration.

By empirical comparison of the amplification curves, the optimal concentration of MgCl2 in the reaction mixture was 3 mM (3 μL of a 25 mM MgCl2 solution in 25 μL of the reaction mixture). Higher concentrations of magnesium ions having an insignificant inhibitory effect, slightly reduce the reaction efficiency by 1-1.5 threshold cycle values (Table 2).

Table 2: Quantitative PCR results using the developed primers and probes for *C. perfringens*, *C. chauvoei*, *C. novyi*, *C. septicum*. HEX-internal control. "-"-negative control

Samples	Cq (FAM)	Cq (HEX)
1 (*C. perfringens*)		+(33.7)
2 (*C. perfringens*)		+(31.8)
3 (*C. perfringens*)		+(31.9)
4 (*C. perfringens*)		+(34.9)
5 (*C. perfringens*)		+(31.9)
6 (*C. perfringens*)	+(27.5)	+(35.3)
7 (*C. perfringens*)	+(21.2)	
8 (*C. perfringens*)	+(22.5)	
9 (*C. perfringens*)	+(23.6)	
10 (*C. perfringens*)	+(19.9)	
-		+(31.2)
1 (*C. chauvoei*)	+(23.1)	+(30.2)
2 (*C. chauvoei*)	+(27.9)	+(32.1)
3 (*C. chauvoei*)	+(21.9)	
4 (*C. chauvoei*)	+(31.6)	+(30.5)
5 (*C. chauvoei*)		+(32.3)
6 (*C. chauvoei*)		+(30.7)
7 (*C. chauvoei*)		+(33.6)
8 (*C. chauvoei*)		+(34.1)
9 (*C. chauvoei*)		+(30.2)
10 (*C. chauvoei*)		+(31.2)
-		+(30,5)
1 (*C. novyi*)	+(22.2)	+(33.2)
2 (*C. novyi*)	+(28.7)	+(33.3)
3 (*C. novyi*)	+(24.5)	
4 (*C. novyi*)	+(30.3)	+(33.9)
5 (*C. novyi*)		+(30.8)
6 (*C. novyi*)		+(34.7)
7 (*C. novyi*)		+(32.9)
8 (*C. novyi*)		+(30.2)
9 (*C. novyi*)		+(33.1)
10 (*C. novyi*)		+(32.5)
-		+(34,7)
1 (*C. septicum*)		+(33.1)
2 (*C. septicum*)		+(32.8)
3 (*C. septicum*)		+(31.5)
4 (*C. septicum*)		+(30.9)
5 (*C. septicum*)		+(31.7)
6 (*C. septicum*)	+(28.5)	+(33.3)
7 (*C. septicum*)	+(22.4)	
8 (*C. septicum*)	+(21.3)	
9 (*C. septicum*)	+(24.6)	
10 (*C. septicum*)	+(20.1)	
-		+(32.5)

As a result, the PCR mixture had the following composition: primer F (10 pmol) 1.5 μL, primer R

(10 pmol) 1.5 μL, dNTP 2 mM 3 μL, BuferKCl 10×3 μL, MgCl2 25 μM-3 μL, TaqPolymeraza 0.2 μL, DNA 5 μL and dionized water. To carry out the PCR-RV, a probe (3.5 pmol).

The results of DNA analysis of *C. perfringens*, *C. chauvoei*, *C. novyi*, *C. septicum* using quantitative PCR using primers for alpha-toxin, phospholipasaeC (plc), flagellin (fliC) are shown in Table 2.

Evaluation of the effectiveness of PCR-RV, the presence of inhibitors in the mixture and the sensitivity of the reaction.

To evaluate the effectiveness, sensitivity of the reaction and the presence of potential inhibitors, a standard procedure was used in the reaction mixture. For this purpose, PCR-PB was carried out for consecutive tenfold dilutions of specific genomic DNA. Each concentration (in the range of 101-106 pg DNA per reaction) was analyzed in four replicates.

The sensitivity of the test systems was about 10 pg of specific DNA per reaction which is about 200 copies, taking into account the size of the genome of bacteria of the genus Clostridium of the order of about 36 million bp. The efficiency of the reaction was 97% in the experiments which is a very high efficiency value for PCR diagnostic systems.

CONCLUSION

As a result of the studies, primers and probes were selected that allow detection of *C. septicum*, *C. perfringens*, *C. chauvoei*, *C. novyi*. The test system has the necessary specificity and sensitivity, excludes false-positive results. The method is based on PCR amplification of DNA fragments that were developed on the basis of the genes: alpha-toxin, phospholipasae C (plc), flagellin (fliC). In addition to providing accurate, reliable and rapid diagnosis of the microorganism, another advantage of this method is that it excludes the use of carcinogens (ethidium bromide), since PCR-Real-time uses fluorescent labeled probes, i.e., no gel Electrophoresis.

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