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Применение молекулярных методов для идентификации *Clostridium sporogenes, Clostridium perfringens* и *Clostridium sordellii,* выделенных от крупного рогатого скота

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Представлены результаты исследований по разработке эффективного метода видовой идентификации клостридий *Clostridium sporogenes, Clostridium perfringens* и *Clostridium sordellii*, основанного на полимеразной цепной реакции (ПЦР), а также возможности его практического использования для диагностики клостридиозов крупного рогатого скота. Всего исследовали 90 проб биологического материала, отобранных от больных животных в хозяйствах Новосибирской области в 2023 г., которые изучали бактериологическим и биохимическим методами. ПЦР проводили с детекцией в реальном времени. Полученные результаты подтверждали секвенированием ПЦР-фрагментов гена 16S рРНК. В результате бактериологических исследований, изучения культурально-морфологических и биохимических свойств идентифицировали 44 изолята клостридий, принадлежащих к девяти видам. Выбраны три пары праймеров и зондов для амплификации фрагментов генов gerKA *C. sporogenes,* plc *C. perfringens* и NanS *C. sordellii*. В результате экспериментальных исследований определили рабочие концентрации праймеров и зондов, обеспечивающих необходимую чувствительность метода, оптимизировали условия проведения ПЦР. Специфичность выбранных олигонуклеотидов проверяли с использованием штаммов клостридий других видов и бактерий других видов. Чувствительность ПЦР составила при исследовании чистых культур бактерий не менее 10^2 КОЕ/мл, суспензий из проб биоматериала – не менее 10^5 КОЕ/мл. В сравнении с непосредственным исследованием проб биоматериала в смешанных культурах бактерий, выделенных из этих же проб на питательных средах, количество положительных результатов было выше (53 и 37% соответственно). Разработанный метод позволяет в более короткие сроки и эффективнее проводить диагностику клостридиозов крупного рогатого скота. Чувствительность разработанного метода выше при исследовании бактериальных культур, выделенных на питательных средах, чем при непосредственном исследовании проб биоматериала. Последующее более детальное изучение молекулярно-генетических характеристик выявленных изолятов клостридий на наличие генов токсинов позволит определить их потенциальную токсигенность и роль в развитии болезни.

Ключевые слова: крупный рогатый скот, клостридиозы, полимеразная цепная реакция, диагностика, колониеобразующие единицы, *Clostridium sporogenes, Clostridium perfringens, Clostridium sordellii*

Application of molecular methods for the identification of *Clostridium sporogenes*, *Clostridium perfringens* and *Clostridium sordellii* isolated from cattle

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The results of research on the development of an effective method of species identification of *Clostridium sporogenes*, *Clostridium perfringens* and *Clostridium sordellii* based on polymerase chain reaction (PCR), as well as the possibility of its practical use for the diagnosis of clostridiosis in cattle are presented. A total of 90 samples of biological material collected from diseased animals in the farms of the Novosibirsk region in 2023 were studied by bacteriological and biochemical methods. PCR was performed with real-time detection. The obtained results were confirmed by sequencing of PCR fragments of 16S rRNA gene. As a result of bacteriological studies, culture-morphological and bio-

chemical properties, 44 isolates of clostridia belonging to nine species were identified. Three primer and probe pairs were selected for the amplification of the gene fragments gerKA *C. sporogenes*, plc *C. perfringens*, and NanS *C. sordellii*. As a result of experimental studies, the working concentrations of primers and probes, providing the necessary sensitivity of the method, were determined and the conditions of PCR were optimized. Specificity of the selected oligonucleotides was tested using the strains of other species clostridia and bacteria of other species. The sensitivity of PCR was at least 10^2 CFU/ml for pure bacterial cultures and at least 10^5 CFU/ml for suspensions from biomaterial samples. Compared to direct examination of the biomaterial samples in mixed cultures of bacteria isolated from the same samples on nutrient media, the number of positive results was higher (53 and 37%, respectively). The developed method allows for shorter and more effective diagnostics of bovine clostridiosis. Sensitivity of the developed method is higher when examining the bacterial cultures isolated on nutrient media than when directly examining biomaterial samples. Subsequent more detailed study of molecular genetic characterization of the identified clostridium isolates for the presence of toxin genes will help to determine their potential toxigenicity and role in disease development.

Keywords: cattle, clostridioses, polymerase chain reaction, diagnosis, colony-forming units, *Clostridium sporogenes, Clostridium perfringens, Clostridium sordellii*

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INTRODUCTION

In recent years, the frequency of clinical manifestation of clostridiosis in cattle has increased in the Russian Federation. It is caused by intensification of animal husbandry, aimed at increasing milk productivity of cows, which is often carried out against the background of unbalanced feeding rations. This leads to metabolic disorders, in particular ketosis, acidosis, and in most cases to the death of animals. Multiplying on the mucous membrane of the gastrointestinal tract, clostridia cause necrosis of epithelial cells, which contributes to the penetration of toxins produced by them into the bloodstream and the development of severe pathological processes in the body [1, 2]. Due to the rapidity of the infectious process, high toxigenic activity of pathogens and extensive damage to organs and tissues of the body, the treatment of such animals is often futile and highly productive animals have to be culled [3, 4].

Clostridia are spore-forming anaerobic Gram-positive bacteria of the genus *Clostridium*, family *Clostridiaceae*, order *Clostridiales*, class *Clostridia*, supraclass *Clostridium*, type *Fusobacteria*, subkingdom *Bacteria* – they are widely distributed in soil, water, decomposition (putrefaction) products of protein substances. The genus Clostridium has more than 100 species. Most of these species are the representa-

tives of normal microflora of the gastrointestinal tract of cattle. However, some of them are pathogenic and produce exotoxins that act locally or systemically[1] [5].

Diseases caused by toxigenic clostridia are conventionally divided into three main types: neurotoxic (botulism, tetanus); histotoxic (clostridial myositis or "black leg", gas gangrene, malignant edema, bacillary hemoglobinuria, infectious necrotic hepatitis); intestinal (caused by *C. perfringens* types A, B, C, D and E, necrotic enteritis, diarrhea caused by *C. difficile*, clostridium abomasit, etc.) [6].

The greatest harm to cattle is caused by histotoxic clostridia, which include *C. chauvoei* (quarter evil), *C. novyi* (*C. oedematiens*) type A, *C. septicum, C. sordellii* (malignant edema), *C. haemolyticum* (*C. novyi* type D) (bacillary hemoglobinuria), *C. novyi* type C (chronic osteomyelitis of buffalos), *C. perfringens* type A (malignant edema, gas gangrene, enteritis necroticans, metritis, cattle mammitis), *C. septicum, C. oedematiens* (*C. novyi*), *C. histolyticum* (malignant edema, bradsot-like cattle infections) [1, 6].

In addition to the above-described species of clostridia, other species can be isolated from cattle, many of which under favorable conditions can also synthesize toxins and cause the relevant pathology.

When examining slaughter products from healthy animals, the most commonly identified are *C. sporogenes* (13%), *C. cadaveris* (12,5), *C. cochlearium* (12) and *C. perfringens* (10), C. *sordellii* (8%) [7]. Of them *C. sporogenes*, *C. cadaveris*, *C. cochlearium* are representatives of the normal intestinal microflora. Only in rare cases are they able to cause the development of purulent infection or enhance the pathogenicity of other clostridia, whereas *C. perfringens* and *C. sordellii* are recognized as cattle pathogens [8, 9].

Species identification of clostridia is necessary to make a correct diagnosis.

Currently, bacteriological and biochemical methods remain the main methods of clostridium species identification. Their disadvantages include the duration of analysis (at least 14 days), as well as the similarity of cultural, morphological and biochemical properties of some clostridium species.

Methods of species identification of pathogens based on the detection of genome fragments in the sample are devoid of these disadvantages. Currently, foreign researchers have developed various PCR variants to detect both individual clostridia species and to identify the selected isolates by pathogenicity factors [10, 11]. Commercial kits to detect the bacterium *Clostridium perfringens* and the toxins it produces and for the identification of *Clostridium difficile* ("Vector-Best" company) in animal biomaterial samples by real-time PCR method are made in the Russian Federation.

Often other Clostridium species in combination with each other are involved in the etiology of diseases in cattle.

The purpose of this work is to develop a method for detection and identification of clostridium species *C. sporogenes, C. perfringens* and *C. sordellii* based on PCR, as well as its practical use for the diagnosis of bovine clostridiosis in cattle.

MATERIAL AND METHODS

In the period 2022, 2023 90 samples of internal organs (muscle tissue, liver, mediastinal and mesenteric lymph nodes, lungs) from animals of different ages from the farms of Western Siberia were investigated. Samples of biological material were taken no later than 4 h after the death or slaughter of the animal, immediately cooled and delivered to the laboratory for examination within 12 h.

[1]*Silva R.O.S., Uzal F.A., Oliveira Jr C.A., Lobato F.C.F.* Clostridial Diseases of Animals // John Wiley & Sons, Ltd.; Hoboken, NJ, USA. Gangrene Gas (Malignant Edema), 2016, pp. 243–254. DOI: 10.1002/9781118728291.ch 20.

Before the study, biomaterial samples were ground in porcelain mortars and resuspended in 70% ethyl alcohol at a ratio of 1: 5 – 1: 10, then centrifuged. After that, the precipitate was transferred to a tube with 5 ml of thioglycol medium, cultured in anaerobic conditions for 7–10 days at 37 ° C. After 7 days of incubation, the tubes were periodically reviewed and those in which a change or clouding of the medium was observed were selected. Then these tubes were heated for 10 min at 80 °C and the contents were sown on meat-peptone agar with 5% defibrinated ram's blood and clostridial agar (Himedia, India), incubated for 24-48 h at 37 °C in an atmosphere of 10% CO_2.

After 24–48 h of incubation, agar dishes were examined for the presence of typical colonies (up to 3 mm in diameter, rounded with even or indented edges, mucous consistency, gray in color, with the presence of ß-hemolysis zone and ultraviolet luminescence), and then micromorphology was studied in Gram-stained smears. If Gram-positive bacilli with spores located centrally or subterminally were detected, bacterial suspensions were taken for PCR study.

In parallel, bacterial suspensions were examined by biochemical method using the ANAEROTest 23 anaerobic bacteria identification kit (Erba Mannheim, Czech Republic).

In PCR staging, bacterial isolates of *C. sordellii* (T2308), *C. perfringens* (T2304), and *C. sporogenes* (K2301), previously identified by bacteriological and biochemical methods, were used as positive control samples, the results of which were confirmed by 16S rRNA sequencing.

Before the study, the bacterial suspension was heated at 100 °C for 10 min. DNA extraction was performed using the Ribo-prep kit (Amplisens, Federal Budget Institute of Science "Central Research Institute of Epidemiology") according to the manufacturer's instructions. Then 5 µl of the obtained supernatant was used for PCR performance.

To identify genetic material in bacteria *C. sporogenes*, *C. perfringens* and *C. sordellii* primers and probes were selected by real-time PCR (see Table 1).

The PCR reaction mixture included PCR buffer (60 mM Tris-HCl [pH 8.5], 1.5 mM $MgCl_2$, 25 mM KC1, 10 mM 2-mercaptethanol, 0.1% Triton X-100), 0.2 mM dNTP, 0.2 µg of each primer and probe, 1.25 e.a. of Taq-DNA polymerase, and 5 µL of extracted DNA.

Табл. 1. Структура праймеров и зондов для идентификации *C. sporogenes, C. perfringens* и *C. sordellii*

Table 1. Structure of primers and probes for identification of *C. sporogenes, C. perfringens* and *C. sordellii*

Bacterium	Primer and probe	Sequence (5' → 3')	Target gene	Amplicon size, bps.
C. sporogenes	ClsporF ClsporR ClsporZ	5-CAGTGTGGTGGGTGGTATTAT -3 5- GCCACTGTAGAAACACCTACT -3 5(HEX)- TAGGTGATGCAGCCATAAGGGCAA -3(BHQ1)	gerKA	96
C. perfringens	ClperfF ClperfR ClperfZ	5- GCTAGATATGAATGGCAAAGAGG -3 5- CGGCAGTAACATTAGCAGGA -3 5(FAM)- AGCTACATTCTATCTTGGAGAGGCTATGCA -3(BHQ1)	plc	115
C. sordellii	ClsordF ClsordR ClsordZ	5- TCAGACTTTGGCAGATGGTACTATG -3 5- AGTCCCATGTTTGTCCATTATCAGT -3 (ROX)- TGGAGCAGMRGATCATGCATACAT -(BHQ2)	NanS	119

For PCR the following temperature conditions were used: 95 °C – 5 min, 1 cycle; 95 °C – 15 s, 60 °C – 60 s, 45 cycles. Fluorescence was measured at 60 °C on all channels. Samples with Ct value (cycle threshold) not exceeding 39 were considered positive.

PCR for amplification of the16S rRNA gene fragment was performed with primers SJ-F (679) CGGTGAAATGCGTAGAKATTA, SJ-R (952) CGAATTAAACCACCACATGCTCCG to amplify a 273-bp fragment[2].

The nucleotide sequence of the DNA fragments was determined using BigDye® Terminator v3.1 Cycle Sequencing Kits (Applied Biosystems, USA). Secretion reaction products were analyzed by capillary electrophoresis in an ABI PRISM® 3130xl automated sequencer (Applied Biosystems/Hitachi, Japan). The nucleotide sequences were aligned using the Lasergene 11 program. The aligned sequences were compared with the sequences of the NCBI BLAST database.

RESULTS AND DISCUSSION

At the first stage of research, the sensitivity of the reaction for the identification of three species of clostridia was determined using isolates of *C. sporogenes*, *C. perfringens* and *C. sordellii* as control samples. For this purpose, suspensions of 24-hour bacterial cultures with a concentration of 10^{11} CFU/ml were prepared, which were titrated by the method of 10-fold dilutions up to a concentration of 10^{1} CFU/ml, 5% suspension of biomaterial samples from animals. The results of determining the diagnostic sensitivity of the developed method are presented in Table 2.

The results of the studies indicate that PCR has high diagnostic sensitivity and allows identification of clostridium species both in bacterial suspensions and in animal organs. The sensitivity of the method for bacterial cultures was higher than for organ suspensions and amounted to 10^{3} CFU/ml for C. sporogenes and 10^{2} CFU/ml for *C. perfringens* and *C. sordellii*.

Previously isolated clostridium isolates as well as bacterial strains *M. haemolytica*, *E. coli*, *P. multocida*, *S. typhimurium* were examined to determine the specificity and efficiency of PCR (see Table 3).

To study the diagnostic efficiency of the developed method, biomaterial samples obtained from sick animals, isolated bacterial cultures, as well as bacteria identified preliminarily by biochemical method using the ANAEROTest 23 anaerobic bacteria identification kit (Erba Mannheim, Czech Republic) were examined (see Table 4).

The study of 90 samples of biomaterial from sick animals by PCR method revealed 37 positive samples. The bacteriological method allowed to isolate bacterial cultures from all biomaterial samples, but the PCR method confirmed the presence of clostridia of the desired species only in 53 of them (*C. sporogenes* – 34, *C. perfringens* – 27, *C. sordellii* – 21). Probably, clostridia of other species were present in other samples. For more detailed studies, 44 isolates of clostridia were selected and preliminarily investigated by a biochemical method.

Табл. 2. Определение диагностической чувствительности ПЦР при идентификации *C. sporogenes, C. perfringens* и *C. sordellii*

Table 2. Determination of the diagnostic sensitivity of PCR in the identification of *C. sporogenes*, *C. perfringens* and *C. sordellii*

Clostridium species	Isolate	Sensitivity, CFU/ml	
		Bacterial culture	Organ suspension
C. sporogenes	K2301	10^{3}	10^{6}
C. perfringens	T2304	10^{2}	10^{5}
C. sordellii	T2308	10^{2}	10^{5}

[2]*Hu X.L., Wang H.Y., Wu Q., Xu Y.* Development, validation and application of specific primers for analyzing the clostridial diversity in dark fermentation pit mud by PCR-DGGE // Bioresource technology, 2014, vol. 163, pp. 40–47. DOI: 10.1016/j.biortech.2014.04.008.

Табл. 3. Определение специфичности реакции при выявлении и генотипировании *C. sporogenes, C. perfringens* и *C. sordellii*

Table 3. Determination of the specificity of the reaction in the detection and genotyping of *C. sporogenes*, *C. perfringens* and *C. sordellii*

Sample	Ct value		
	JOE/HEX channel (*C. sporogenes*)	FAM channel (*C. perfringens*)	ROX/Texas Red channel (*C. sordellii*)
C. sordellii (isolate T2308)	Negative	Negative	13,35
C. perfringens (isolate T2304)	»	16,28	Negative
C. sporogenes (isolate K2301)	15,37	Negative	»
Mannheimia haemolytica (strain 16)	Negative	»	»
Pasteurella multocida (strain T80)	»	»	»
Pasteurella multocida (strain 681)	»	»	»
Salmonella typhimurium (strain 1891)	»	»	»
Escherichia coli (strain F-50)	»	»	»

Табл. 4. Сравнительная диагностическая эффективность ПЦР

Table 4. Comparative diagnostic effectiveness of PCR

Research method	Samples examined	Research results		
		C. sporogenes	*C. perfringens*	*C. sordellii*
PCR of biomaterial samples from animals	90	14	13	10
PCR of bacterial cultures	90	34	27	21
PCR of bacterial cultures examined by biochemical method	44	11	11	10

C. sporogenes was detected in 11 of them, *C. perfringens* in 11, and *C. sordellii* in 10. Nine isolates that were negative by PCR were assigned to species by16S rRNA sequencing: *C. histolyticum* – 4, *C. haemolyticum* – 1, *C. novyi* – 1, *C. oedematiens* – 1, *C. aerotolerans* – 1, *C. pupuleti* – 1.

In the study of biomaterial samples from animals by PCR method, a greater number of negative samples for the presence of clostridia was obtained than in the analysis of bacterial cultures isolated from these samples. This difference is probably due to the low concentration of clostridia in animal organs.

C. perfringens is one of the most common bacteria that can be isolated from soil samples, intestinal contents of healthy humans and animals [12], but under certain conditions it can cause enteritis and abomasitis in cattle[3]. *C. sordellii* more often can be the cause of sudden death of sheep of all ages, acute abomasitis of calves and lambs, hemorrhagic enteritis, gangrenous lesions of reproductive tract organs of new cows. It may be present in wounds in asso-

[3]*Goossens E., Valgaeren B.R., Pardon B., Haesebrouck F., Ducatelle R., Deprez P.R., Van Immerseel F.* Rethinking the role of alpha toxin in Clostridium perfringens-associated enteric diseases: a review on bovine necro-haemorrhagic enteritis // Veterinary research, 2017, vol. 48 (1), p. 9. DOI: 10.1186/s13567-017-0413-x.

ciation with other anaerobic and aerobic bacteria[4]. *C. sporogenes* is more often a commensal of the intestinal microbiome, but can sometimes cause a septic form of infection [13].

As a rule, outbreaks of clostridiosis in cattle at farms are most often caused by associations of bacteria. For example, the development of malignant edema may simultaneously involve *C. perfringens, C. oedematiens, C. septicum, C. sordellii*, *C. histolyticum.* Mixed type diseases are clinically more severe, acute and usually end in the death of the animal[5, 6].

In our studies, *C. perfringens* and *C. sporogenes* were most often present in biomaterial samples from sick animals, and less frequently *C. sordellii* was present in different combinations.

CONCLUSION

The developed PCR method has high sensitivity, specificity and allows increasing the efficiency of diagnostic tests for bovine clostridiosis by reducing the time of their realization. This method can be used in diagnostic laboratories to detect clostridia of *C. sporogenes*, *C. perfringens* and *C. sordellii* species in biomaterial samples from animals and in bacterial cultures. The isolated and identified bacterial cultures can be used in the future to study their molecular genetic properties, including the presence of genes responsible for toxin production to determine their toxigenic potential and role in disease development.

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