Improvement of Laboratory Diagnosis for Detection and Identification of Bovine Clostridiosis

NATALIA A. BEZBORODOVA*, EVGENIA N. SHILOVA,
VERONIKA V. KOZHUKHOVSKAYA, VLADLENA D. ZUBAREVA, OLGA V. SOKOLOVA,
NIKOLAI A. MARTYNOV
Federal State Budgetary Scientific Institution "Ural Federal Agrarian Scientific Research Centre, Ural Branch of Russian Academy of Sciences"
Ekaterinburg
RUSSIAN FEDERATION

**Corresponding Author*

Abstract: - **Objective:** Clostridiosis is a toxic infectious disease; the pathogenicity factor of causative agents is the secreted toxins. A characteristic feature of clostridiosis pathogens is their polytropism. They affect both humans and agricultural, domestic, and wild animals. Our research aimed to monitor *Clostridium perfringens* and *Clostridium difficile* spread among agricultural organizations of the Ural region.
Materials and Methods: 137 biological samples were obtained from cattle with symptoms of clostridial infection. For PCR species and toxinotype identification commercial kits and previously described protocols were used. Results verification was conducted using MALDI-TOF MS.
Results: Out of 137 samples of selected material *Clostridium* was detected in 40.6% of samples: *Cl. difficile* in 35.8%, *Cl. perfringens* in 25.3%, *Cl. difficile*+*Cl. perfringens* in 16.4%. *Cl. difficile* and *Cl. perfringens* were found in 30.5% of fecal samples, in pathological material from dead calves and cows – 8.7%, in milk samples – 1.4%.
Conclusion: Laboratory methods made it possible to verify the diagnosis: infectious anaerobic enterotoxemia of calves in one case, necrotic enteritis in 3 animals, and intestinal toxic infection caused by *Cl. perfringens* type A in 2 cows and 5 calves. The diagnostics of toxinotypes of *Cl. perfringens* have made it possible to conduct toxin-specific vaccination against clostridial infection in farms.

Key-Words: - cattle, Clostridium difficile, Clostridium perfringens, PCR, toxinotypes.

Received: August 2, 2022. Revised: October 6, 2023. Accepted: October 21, 2023. Published: November 1, 2023.

1 Introduction

Bovine clostridiosis in cattle is one of the major problems in veterinary medicine, animal husbandry, and food safety. This pathogen causes infectious diseases related to zooanthroponosis diseases, [1], [2], [3], [4], [5].

Anaerobic spore-forming bacteria of the genus *Clostridium* cause infections, which occur ubiquitary. Specific features of this group of diseases are stationarity and high mortality. Generally, the disease manifests itself in sporadic form, sometimes as an epidemic outbreak. The main reservoir of these anaerobes is soil, as well as the intestines of animals and humans, [1], [6]. Under certain conditions clostridia, which normally inhabits the gastrointestinal tract, can gain pathogenicity properties. Metabolic disorders, stress factors, injuries, and misuse of antibacterial drugs contribute to the spreading of toxigenic anaerobes, [7], [8], [9].

Active exotoxins are the main factors of clostridiosis pathogenesis. The most common zoonotic pathogens of clostridial infections include *Clostridium perfringens,* [6], [10]. There are five toxinotypes of this pathogen: A, B, C, D, and E, each of which causes a disease with specific clinical signs, [11]. All toxinotypes cause anaerobic enterotoxemia in young animals, [8], [10]. Toxinotype A causes malignant edema, and gas gangrene in cows with injuries. *Cl. perfringens* toxinotype A causes less malignant diseases than other toxinotypes. Mortality of animals with toxinotype A-caused infection does not exceed 25%, [2], [11], [12], [13]. Toxinotype B provokes enterotoxemia in calves with hemorrhages in all vital organs. *Cl. perfringens* toxinotype C causes necrotizing enteritis in animals. This toxin causes

intestinal necrosis, bleeding, septic shock, and eventually death, [14], [15]. *Cl. perfringens* type D is another pathogen that causes enterotoxemia in young ruminants with specific pathological findings also known as pulpy kidney, [16], [17]. Toxinotype D causes dysentery and nephrolithiasis in sheep and lambs, [18]. In calves it causes enterotoxemia with neurological signs, without extensive intestinal lesions. Toxinotype E is the cause of necrotic hemorrhagic enteritis in calves, [19].

Cl. difficile is a gram-positive toxin-producing bacterium. Being an intestinal pathogen it is capable of releasing enterotoxin A, cytotoxin B, and binary toxin CDT. Toxins A and B are encoded by separate genes (*tcdA*, *tcdB*), while the binary CDT toxin is encoded by two genes (*cdtA* and *cdtB*), [10], [20]. Toxin A causes pronounced enterotoxic manifestation and pro-inflammatory activity of interleukins in intestinal epithelial cells. Toxin B triggers receptor-mediated endocytosis, [21]. Clostridia that produce these toxins have increased adhesion to the intestinal epithelium, [4], [22]. Toxin-negative isolates of *Cl. difficile* are often detected in the feces of animals and humans with intestinal dysbiosis. The acquisition of toxigenic properties in initially toxin-negative isolates of *Cl. difficile* can occur through horizontal gene transfer, [4], [23].

The species composition of the genus *Clostridium*, which provokes diseases in livestock farms, has not been studied enough, especially in the Russian Federation. There are reports of bovine clostridiosis being caused by seven species of the genus *Clostridium* with a predominance of *Cl. perfringens*, *Cl. septicum*, *Cl. sporogenes,* [24], [25]. It is necessary to know causative species to improve the effectiveness of preventive measures against clostridial infections in cattle. Currently, several methods of laboratory diagnostics of *Clostridium spp*. are used: culture, enzyme immunoassay, immunochromatographic analysis, and polymerase chain reaction, [26], [27]. Only one commercial PCR kit for the detection of *Cl. perfringens* is available in the Russian Federation. Yet, several commercial kits allow simultaneous detection of toxinotypes: BactoReal® Kit Clostridium perfringens (Austria), and RIDA®GENE Clostridium difficile (Germany). The development of new extended assays that allow genotyping of clostridial pathogens is an important task for the development of an effective strategy for the elimination and prevention of these dangerous, often incurable diseases, [4], [11].

The purpose of this study was to improve laboratory diagnosis for the detection and identification of clostridium infections in cattle. To do that monitoring for the spread of *Cl. difficile* and *Cl. perfringens* toxinotypes on farms of the Ural region was conducted.

2 Materials and Methods

2.1 Ethical Approval

The institutional ethics committee of the Federal State Budgetary Scientific Institution “Ural Federal Agrarian Scientific Research Centre, Ural Branch of Russian Academy of Sciences” approved this study with protocol number: 515.

2.2 Sample Collection, Isolation and Identification

Overall, in 2023, 137 samples of biological material from cows and calves (*Bos taurus*) from 21 agricultural organizations of the Ural region were studied. Collected biomaterial included: feces, milk, and swabs from the wounded hooves. Samples of pathological material from dead calves and cows included: the heart, liver, kidneys, spleen, lungs, rumen, abomasum, and reticulum.

The Diatom DNA Prep 200 kit (IsoGen LLC, Moscow) was used to extract DNA from the biomaterial. The HiPure Stool DNA Kit (Guangzhou Magen Biotechnology Co., Ltd (Magen), China) was used to obtain high-quality DNA from feces. Assay kits "RealBest-Vet DNA Cl.difficile/Cl.perfringens" were used to detect clostridial infection in the biomaterial. RealBest-Vet DNA Cl.difficile tcdA/tcdB/CDT kit (JSC Vector-Best, Moscow) was used for typing *Cl. difficile*. Amplification with real-time detection was performed using QuantStudio 5 (USA). MALDI-TOF mass spectrometry verification of the results was conducted on a VITEK MS analyzer (bioMerieux SA, France) in the laboratory of Quality Med LLC (Ekaterinburg).

2.3 Molecular Identification of *etx*, *iap*, *plc*, *cpe*, and *cpb* Genes by PCR

Toxinotypes *Cl. perfringens* was determined by PCR based on the presence of the *etx*, *iap*, *plc*, *cpe* and *cpb* genes. Genotyping was carried out according to the protocol proposed by Julian I. Rood, [11], Primers’ sequence are shown in **Table 1**. 10 µl reaction mix included: SibEnzyme SE-Buffer (60 mM Tris-HCl (pH 8.6), 25 mM KCl, 10 mM 2-mercaptoethanol, 0.1% Trion X-100), 0.18

mM of each dNTP , 1.16 mM MgCl2, 0.06 units of Taq-polymerase (LLC SibEnzyme, Russia); 15-40 ng of DNA, 0.35 μM of each primer (LLC DNA-Synthesis, Russia).

Table 1. Primers' sequence for *etx*, *iap*, *plc*, *cpe* and *cpb* genes detection

Gene	Primers' sequence (5'-3')	Amplicon
etx	ETX_F-CCACTTACTTGTCCTACTAAC	656 b.p.
	ETX_R-GCGGTGATATCCATCTATTC	
iap	IAP_F-GGAAAAGAAAATTATAGTGATTGG	461 b.p.
	IAP_R-CCTGCATAACCTGGAATGGC	
plc	PLC_F-GGACCAGCAGTTGTAGATA	324 b.p.
	PLC_R-CCTCTGATACATCGTGTAAG	
cpe	CPE_F-GGAGATGGTTGGATATTAGG	233 b.p.
	CPE_R-GGACCAGCAGTTGTAGATA	
cpb	CPB_F-GCGAATATGCTGAATCATCTA	196 b.p.
	CPB_R-GCAGGAACATTAGTATATCTTC	

Amplification protocol: preliminary denaturation at 95 °C – 5 minutes; further 35 cycles: denaturation at 94°C – 30 seconds, primer annealing at 60°C – 30 seconds, elongation at 72°C – 30 seconds, final elongation at 72°C – 10 minutes. The result of amplification was evaluated by electrophoresis in 3% agarose gel. *Clostridium perfringens* ATCC 13124 type A (BD Microtrol, USA) was used as a positive control.

Statistical data processing was carried out using Microsoft Office Excel 2019.

3 Results

PCR results showed that *Clostridium* DNA was found in 40.6% out of 137 samples. *Cl. difficile* was detected in 48 samples (35.8%). *Cl. perfringens* was found in 34 samples (25.3%). The simultaneous presence of *Cl. difficile* and *Cl. perfringens* was observed in 16.4% of samples. *Cl. difficile* and *Cl. perfringens* were found both in feces (30.5%) and in pathological material from dead calves and cows (8.7%), as well as in milk samples (1.4%) (Figure 1).

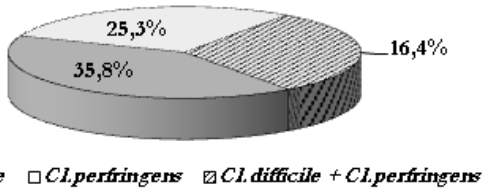

Fig. 1: *Clostridium* is detected from biological material from cows.Electrophoresis was used for the detection of the *etx*, *iap*, *plc*, *cpe,* and *cpb* genes in *Cl. perfringens* (Figure 2).

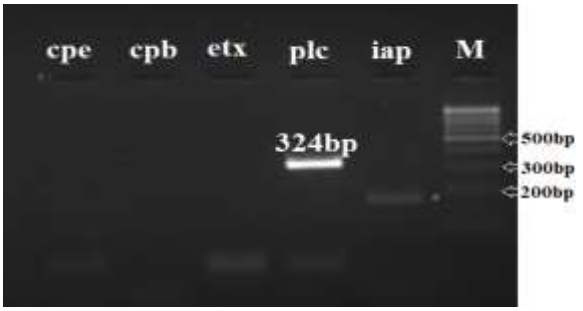

Fig. 2: Results of amplicon electrophoresis for genotyping *Cl. perfringens* toxinotype A. Designations: *plc* gene (324 b.p.); *etx*, *iap*, *cpe*, *cpb* are *Cl. perfringens* toxin genes; M is a size standard with a step of 100 b.p.

Cl. perfringens with the *plc* gene is responsible for the production of α-toxin. It was detected by PCR in 6 fecal samples from cows and calves that had signs of diarrhea. Also, *plc*, *etx*, *cpe* genes were detected in 3 fecal samples from calves. These genes are responsible for the production of α, ε-toxins and enterotoxin, respectively. Those isolates belong to toxinotype D. *Cl. perfringens* toxinotype C (*plc*, *cpb*) was detected in some fecal samples. *Cl. perfringens* toxinotype A was detected in parenchymal organs of the dead calf.

Cl. difficile was toxigenic in 56.2% of isolates. The genes of binary toxin (CDT) were found most often – in 70.0% of isolates, genes of toxin B – in 51.8% and toxin A – in 48.1% of isolates. Several *Cl. difficile* toxinotypes were detected simultaneously: toxins A + B + CDT in 18.5% isolates, A + CDT in 14.8% isolates, and few isolates had A + B and B + CDT combination. Also, non-toxigenic *Cl. difficile* was found in 43.7% of samples.

Overall, toxigenic *Cl. difficile* (68.7% isolates) was detected in calves and cows with diarrhea, which could indicate the presence of an acute intestinal infection in animals. It is known that *Cl. difficile* is the leading cause of antibiotic-associated diarrhea. The genes (*TcdA*, *TcdB*, *CDI*) responsible for the production of toxins are the main virulence factors, [28]. Toxins secreted by *Cl. difficile* damage the intestinal epithelium in young animals, which leads to inflammation, tissue damage, production of pro-inflammatory cytokines in the macroorganism, and the development of the disease, [29].

Cl. difficile was present in 20.8% samples of pathological material from cows and calves. At the same time, *Cl. difficile* was always present in samples with several types of microorganisms (*Escherichia coli*, *Salmonella enterica*, *Cl. perfringens*), which may also indicate a septic process (Figure 3, and Figure 4).

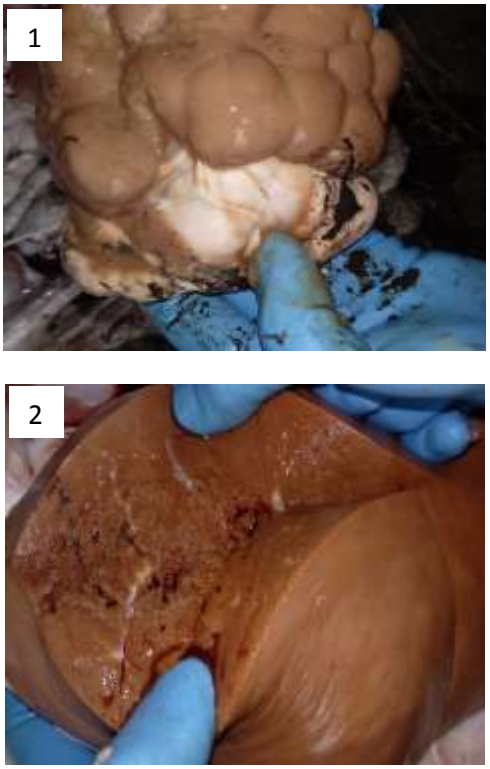

Fig. 3: Sample preparation of pathological material of calves with suspected clostridial infection (1 – kidneys, 2 – liver).

Fig. 4: Sample preparation of pathological material from cows (1 – lung, 2 – spleen, 3 – lymphatic nodes), with suspected mixed bacterial infection (*E. coli, S. enterica, Cl. perfringens, Cl. difficile)*.

Cl. difficile (31.2%), and *Cl. perfringens* (12.5%) were found with the simultaneous presence of *Staphylococcus spp.*, *E. coli*, *Streptococcus agalactiae* and *Staphylococcus aureus* in 16 milk samples from cows with signs of mastitis. In a few samples, toxinotypes of *Cl. difficile* A, B, CDT were detected and the rest of the anaerobes were toxin-negative.

4 Discussion

The species composition of bacteria of the genus *Clostridium*, which causes pathology in cattle in the livestock farms of the Russian Federation, has not been studied enough. Determination of toxins produced by a particular strain or isolate (*Cl. perfringens*, *Cl. difficile*) is important for predicting the course of the disease. In addition, the typing of bacteria of the *Clostridium* genus is an urgent task for a deeper understanding of the epizootic process and should be taken into account when developing vaccine-preventive measures in agricultural enterprises, [30].

The most common pathogenic agent affecting humans and animals is *Cl. perfringens*. Many years of experience of cultivating anaerobes has shown that isolation of *Clostridium* by microbiological methods is very difficult, since they require strict anaerobic conditions. Moreover, samples are often contaminated with a mixed bacterial flora, which complicates the diagnosis. The standard *Cl. perfringens* toxinotype identification by toxins neutralization with specific sera is very laborious, expensive, and time-consuming. Before biochemical identification, it is important to have a pure culture of the strain, which is an arduous process, [31]. PCR has expanded the possibilities of studying clostridial infections in animals with its exceptional sensitivity, specificity, and fast laboratory analysis, [31]. An important advantage of the PCR method is bypassing the stage of cultivation. Real-time PCR studies have opened up a wide range of possibilities in the field of quantitative analysis of bacterial nucleic acids. Currently, only one commercial PCR test system is available in the Russian Federation, which detects the DNA of *Cl. perfringens* without determining the genes responsible for the production of toxins.

Therefore, the development of a test system for the identification of various types of *Cl. perfringens* by molecular genetic methods is very promising. However, in the course of work on the test system, a number of problems arose when working with such biological material as animal feces. Those samples contain many foreign microorganisms and substances that inhibit the PCR reaction (calcium salts and phosphates, bile salts, bilirubins, polysaccharides, undigested plant fibers, mucus, and insoluble products of the gastrointestinal tract), and therefore obtaining pure and high-quality DNA is difficult, [32]. It is also worth noting that some genes encoding toxins are localized on plasmids, which are not permanent inclusions in the cell, and may be lost in the course of obtaining a pure culture, leading to false negative results. Therefore, to obtain high-quality DNA for our work, we used a specialized HiPure Stool DNA Kit (Guangzhou Magen Biotechnology Co., Ltd (Magen), China) designed to isolate nucleic acids from fecal samples. Current test system requires electrophoretic visualization of obtained results and has its drawbacks. They include high probability of contamination, increased requirements for the PCR laboratory, and work with ethidium bromide. To overcome the abovementioned drawbacks we will improve this test system by developing real-time PCR detection of *Cl. perfringens* toxins. Novel test systems will be created to identify significant bacteria of the *Clostridium* genus (*Cl. perfringens*, *Cl. difficile*, *Cl. chauvoei*, *Cl. histolyticum*, *Cl. sordellii*, *Cl. septicum*, *Cl. novyi*, *Cl. tetani*, *Cl. botulinum*) and detect antibiotic resistance genes. Using the developed test systems, an analysis of the species diversity of clinical isolates of *Clostridium* circulating in dairy farms will be carried out. Preventive measures for the clostridial infections in dairy herds will be improved taking into account conducted studies. The obtained data will be used to develop appropriate schemes of vaccination and effective therapy against this dangerous anaerobic infection in agricultural organizations.

5 Conclusion

Out of 137 samples of obtained biological material *Clostridium* was detected in 40.6% of samples: *Cl. difficile* in 35.8%, *Cl. perfringens* in 25.3%, *Cl. difficile+Cl. perfringens* in 16.4%. *Cl. difficile* and *Cl. perfringens* were found in 30.5% of fecal samples, in pathological material from dead calves and cows – 8.7%, in milk samples – 1.4%.

The complex diagnostics of individual cases of clostridiosis in cattle in agricultural organizations of the Ural region was conducted. Epizootic data, clinical signs, and characteristic pathological changes were taken into account. Utilized laboratory methods made it possible to verify infectious anaerobic enterotoxemia in one case, necrotic enteritis in 3 animals, and intestinal toxicoinfection caused by *Cl.perfringens* type A in 2 cows and 5 calves.

Conducted PCR studies showed the presence of various toxinotypes of *Cl. difficile* and *Cl. perfringens*. In some samples, a mixed infection was identified, which included aerobic and anaerobic bacteria. The diagnostics of toxinotypes of *Cl. perfringens* have made it possible to conduct toxin-specific vaccination against clostridial infection in farms. For example, farms were

provided with recommendations for changing the type of vaccine according to diagnostic results.

List of Abbreviations:
PCR, Polymerase chain reaction; MALDI-TOF MS, Matrix-assisted laser desorption ionization time-of-flight mass spectrometry; CDT, Cytolethal distending toxin; DNA, Deoxyribonucleic acid.

Acknowledgments:
The study was carried out within the framework of project No. 23-26-00053 "Development of test systems for molecular genetic diagnostics of Clostridia with identification of toxinotypes and antibiotic resistance genes" with the financial support of the Russian Science Foundation.

References:
[1] Rahman M.T., Sobur M.A., Islam M.S., Ievy S., Hossain M.J., El Zowalaty M.E., Rahman A.T., Ashour H.M. Zoonotic diseases: etiology, impact, and control. *Microorganisms*, Vol. 8 No. 9, 2020, 1405; https://doi.org/10.3390/microorganisms8091405.

[2] Geier R.R., Rehberger T.G., Smith A.H. Comparative genomics of *Clostridium perfringens* reveals patterns of host-associated phylogenetic clades and virulence factors. *Front Microbiol*, Vol. 12, 2021, 649953. http://doi.org/10.3389/fmicb.2021.649953

[3] Xiu L., Liu Y., Wu W., Chen S., Zhong Z., Wang H. Prevalence and multilocus sequence typing of *Clostridium perfringens* isolated from 4 duck farms in Shandong province, China. Poult Sci, Vol. 99, No. 10, 2020, pp. 5105–5117; http://doi.org/10.1016/j.psj.2020.06.046

[4] Fourie J.C.J., Bezuidenhout C.C., Sanko T.J., Mienie C., Adeleke R. Inside environmental *Clostridium perfringens* genomes: antibiotic resistance genes, virulence factors and genomic features. *J Water Health,* Vol. 18, No 4, 2020, pp. 477–493. http://doi.org/10.2166/wh.202 0.029.

[5] Hoelzer K., Bielke L., Blake D.P., Cox E., Cutting S.M., Devriendt B., Erlacher-Vindel E., Goossens E., Karaca K., Lemiere S., Metzner M., Raicek M., Collell Surinach M., Wong N.M,. Gay C., Van Immerseel F., Vaccines as alternatives to antibiotics for food producing animals. Part 1: challenges and needs. *Vet Res,* Vol. 49, No 1, 2018, pp. 64; http://doi.org/10.1186/s13567-018-0560-8

[6] Lobzin Yu.V., Kvetnaya A.S., Skripchenko N.V., Zhelezova L.I., Current notions about etiopathogenic and genetics specific features of *Clostridium perfringens* toxins. *Journal of microbiology, epidemiology and immunobiology*; Vol. 98, 2021, pp. 91–103. http://doi.org/10.36233/0372-9311-37

[7] Hu W.S., Woo D.U., Kang Y.J., Koo O.K., Biofilm and spore formation of *Clostridium perfringens* and its resistance to disinfectant and oxidative stress. *Antibiotics (Basel),* Vol. 10, No 4, 2021, pp. 396; http://doi.org/10.3390/antibiotics10040396.

[8] Gohari I.M., Navarro M.A., Li J., Shrestha A., Uzal F., McClane B.A., Pathogenicity and virulence of *Clostridium perfringens*. *Virulence* Vol. 12, No 1, 2021, pp. 723–753. http://doi.org/10.1080/21505594.2021.1886777.

[9] Simpson K.M., Callan R.J., Van Metre D.C., Clostridial abomasitis and enteritis in ruminants. *Vet Clin North Am Food Anim Pract,* Vol. 34 No 1, 2018, pp. 155–184; http://doi.org/10.1016/j.cvfa.2017.10.010.

[10] Uzal F.A., Navarro M.A., Li J., Freedman J.C., Shrestha A., McClane B.A., Comparative pathogenesis of enteric clostridial infections in humans and animals. *Anaerobe*, Vol. 53, 2018, pp. 11–20. http://doi.org/10.1016/j.anaerobe.2018. 06.002.

[11] Rood J.I., Adams V., Lacey J., Lyras D, McClane BA, Melville SB, Moore RJ, Popoff MR, Sarker MR, Songer JG, Uzal FA, Van Immerseel F. Expansion of the *Clostridium perfringens* toxin-based typing scheme. Anaerobe Vol. 53, 2018, pp. 5–10; http://doi.org/10.1016/j.anaerobe.2018.04.011.

[12] Verherstraeten S., Goossens E., Valgaeren B., Pardon B., Timbermont L., Haesebrouck F., Ducatelle R., Deprez P., Van Immerseel F., Non-toxic perfringolysin O and α-toxin derivatives as potential vaccine candidates against bovine necrohaemorrhagic enteritis. *Vet. J.*, Vol. 217, 2016, pp. 89–94; https://doi.org/10.1016/j.tvjl.2016.09.008.

[13] Kapustin A.V., Laishevtcev A.I., Ivanov E.V., Danilyuk A.V., Species diversity of *Clostridia* causing malignant edema in cattle. *IOP Conference Series: Earth and Environmental Science* Vol. 548 (7), 2020, 072041; http://doi.org/10.1088/1755-1315/548/7/072041.

[14] Posthaus H., Kittl S., Tarek B., Bruggisser J., *Clostridium perfringens* type C necrotic enteritis in pigs: diagnosis, pathogenesis, and prevention. *J Vet Diagn Invest*, Vol. 32, No 2, 2020, pp. 203–212; https://doi.org/10.1177/1040638719900180.

[15] Wagley S., Bokori-Brown M., Morcrette H., Malaspina A., D'Arcy C., Gnanapavan S., Lewis N., Popoff M.R., Raciborska D., Nicholas R., Turner B., Titball R.W., Evidence of *Clostridium perfringens* epsilon toxin associated with multiple sclerosis. *Mult Scler,* Vol. 25, No 5, 2019, pp. 653–660; http://doi.org/10.1177/1352458518767327.

[16] Hussain R., Guangbin Z., Abbas R.Z., Siddique AB, Mohiuddin M, Khan I, Rehman TU, Khan A. *Clostridium perfringens* types A and D involved in peracute deaths in goats kept in Cholistan ecosystem during winter season. *Front Vet Sci,* Vol. 9, 2022, 849856; http://doi.org/10.3389/fvets.2022.849856.

[17] Moustafa S., Zakaria I., Moustafa A., AboSakaya R., Selim A., Bacteriological and serological investigation of *Clostridium perfringens* in lambs. *Sci Rep,* Vol. 12 No 1, 2022, pp.19715 http://doi.org/10.1038/s41598-022-21918-6

[18] 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. *Vet Res,* Vol. 48, No 1, 2017, pp. 9. http://doi.org/10.1186/s13567-017-0413-x.

[19] Sholeh M., Krutova M., Forouzesh M., Mironov S., Sadeghifard N., Molaeipour L., Maleki A., Kouhsari E., Antimicrobial resistance in *Clostridioides (Clostridium) difficile* derived from humans: a systematic review and meta-analysis. *Antimicrob Resist Infect Control,* Vol. 9, No 1, 2020, pp.158. http://doi.org/10.1186/s13756-020-00815-5.

[20] Kachrimanidou M., Tzika E., Filioussis G. *Clostridioides (Clostridium) Difficile* in food-producing animals, horses and household pets: a comprehensive review. *Microorganisms,* Vol. 7, No 12,n 2019, pp. 667. http://doi.org/10.3390/microorganisms7120667.

[21] Venhorst J., van der Vossen J., Agamennone V. Battling enteropathogenic clostridia: phage therapy for *Clostridioides difficile* and *Clostridium perfringens*. *Front Microbiol*, Vol. 13, 2022, 891790. http://doi.org/10.3389/fmicb.2022.891790

[22] Knight D.R., Riley T.V., Genomic delineation of zoonotic origins of *Clostridium difficile*. *Front Public Health*, Vol. 7, 2019, pp. 164. http://doi.org/10.3389/fpubh.2019.00164

[23] Glotova T., Koteneva S.V., Nefedchenko A., Glotov A. Etiological agents causing reproduction pathology in cows in big dairy farms. *Veterinary medicine journal* 3–8, 2023. https://doi.org/10.30896/0042-4846.2023.26.2.03-08.

[24] Danilyuk A.V., Kapustin A.V. The prevalence and species diversity of clostridia, the causative agents of anaerobic infections in cattle. *Trudi VIEV*, Vol. 81, 2019, pp. 19–26. http://doi.org/10.30917/ATT-PRINT-2019-10

[25] Zaragoza N.E., Orellana C.A., Moonen G.A., Moutafis G., Marcellin E. Vaccine production to protect animals against pathogenic *Clostridia*. *Toxins*, Vol. 11, No 9, 2019, 525; https://doi.org/10.3390/toxins11090525.

[26] Lu W., Sun H., Xu Z.-M., Du Z., Si L., Yuan S., Jin J., Jin C.-H. Diagnostic and therapeutic strategy for *Clostridium perfringens* infection in postpartum dairy cows: a report of 14 cases, *Journal of Applied Animal Research*, Vol. 50, No 1, 2022, pp. 350-354; https://doi.org/10.1080/09712119.2022.2078329.

[27] Carter G.P., Chakravorty A., Pham Nguyen T.A., Mileto S., Schreiber F., Li L., Howarth P., Clare S., Cunningham B., Sambol S.P., Cheknis A., Figueroa I., Johnson S., Gerding D., Rood J.I., Dougan G., Lawley T.D., Lyras D. Defining the roles of *TcdA* and *TcdB* in localized gastrointestinal disease, systemic organ damage, and the host response during *Clostridium difficile* infections. *mBio,* Vol. 6, No 3, 2015, e00551; http://doi.org/10.1128/mBio.00551-15.

[28] Chandrasekaran R., Lacy D.B. The role of toxins in *Clostridium difficile* infection. FEMS *Microbiol Rev*, Vol. 41, No 6, 2017, pp. 723–750. http://doi.org/10.1093/femsre/fux048.

[29] Amini M., Kheirkhah B., Shamsaddini Bafti M., Rokhbakhsh Zamin F. Molecular identification and sequence analysis of *Clostridium perfringens* virulence genes isolated from sheep and goats. *Journal of the Hellenic Veterinary Medical Society*, Vol. 74 No 1, 2023, pp. 5345–5354; https://doi.org/10.12681/jhvms.29434.

[30] Ahmed H.A., El Bayomi R.M., Hamed R.I., Mohsen R.A., El-Gohary F.A., Hefny A.A.,

Elkhawaga E., Tolba H.M.N. Genetic Relatedness, antibiotic resistance, and effect of silver nanoparticle on biofilm formation by *Clostridium perfringens* isolated from chickens, pigeons, camels, and human consumers, *Veterinary sciences*, Vol. 9, No 3, 2022, pp. 109. https://doi.org/10.3390/vetsci9030109.

[31] Blau K., Gallert C. Prevalence, antimicrobial resistance and toxin-encoding genes of *Clostridioides difficile* from environmental sources contaminated by feces. *Antibiotics*, Vol. 12, No 1, 2023, pp. 162. https://doi.org/10.3390/antibiotics12010162.

Contribution of Individual Authors to the Creation of a Scientific Article (Ghostwriting Policy)

- N.A. Bezborodova designed the study, conducted PCR, interpreted the data and drafted the manuscript.
- E. N. Shilova contributed in study design and critical checking of manuscript.
- V.V. Kozhukhovskaya was involved in DNA extraction and conducting of PCR.
- V.D. Zubareva contributed in preparing and critical checking of this manuscript.
- O.V. Sokolova contributed in sample collection and critical checking of this manuscript.
- N.A. Martynov helped with PCR protocol optimization.

Sources of Funding for Research Presented in a Scientific Article or Scientific Article Itself

No funding was received for conducting this study.

Conflict of Interest

The authors have no conflicts of interest to declare.

Creative Commons Attribution License 4.0 (Attribution 4.0 International, CC BY 4.0)

This article is published under the terms of the Creative Commons Attribution License 4.0
https://creativecommons.org/licenses/by/4.0/deed.en_US