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Clostridium species diversity in cattle

Pavel N. Shastin, Vasiliy A. Savinov, Aleksey I. Laishevtsev, Ekaterina D. Mandryka, Elizaveta A. Fabrikantova, Anastasia V. Supova
Federal Scientific Centre VIEV, 24/1 Ryazansky prospekt, Moscow 109428, Russia

ABSTRACT

Introduction. Clostridial infections, though relatively sporadic, are globally ubiquitous and specified by high mortality rates, resulting in substantial economic losses to agriculture. In cattle, pathogenic *Clostridia* cause diseases such as enterotoxemia, malignant edema, tetanus, and botulism. The most clinically significant species include *Clostridium septicum*, *Clostridium perfringens*, *Clostridium chauvoei*, *Clostridium novyi*, and *Clostridium sordellii*.

Objective. Study of *Clostridium* spp. diversity by examination of autopsy samples and sections of cattle from different regions of Russia; determination of their anatomical localization as well as antibiotic resistance of *Clostridium perfringens* to the most common groups of antibiotics.

Materials and methods. Throughout the study, we adhered to internationally recognized regulatory frameworks and methodological guidelines, employing standardized microbiological and mass-spectrometric methods. Antibiotic resistance was tested against multiple drug groups, such as macrolides, monobactams, penicillins, polypeptides, glycopeptides, aminoglycosides, carbapenems, lincosamides, tetracyclines, ansamycins, diaminopyrimidines, fusidic acid derivatives, etc. *Clostridium* isolates were recovered and identified using routine bacteriological methods coupled with MALDI-ToF mass spectrometry.

Results. Analysis of 359 biological samples resulted in isolation and identification of 137 *Clostridium* isolates (*Paraclostridium bifermentans*, *Clostridium perfringens*, *Clostridium tertium*, *Clostridium butyricum*, *Clostridium septicum*, *Clostridium sporogenes*, *Clostridium cadaveris*, *Clostridium sphenoides*, *Clostridium cochlearium*, *Clostridium sartagoforme*, *Clostridium chauvoei*, *Clostridium novyi*, *Clostridium sordellii*, *Clostridium paraputrificum*, *Clostridium* spp.), of which 25 exhibited pathogenic potential and 17 demonstrated toxigenic properties. *Clostridia* were most frequently isolated from the liver, small and large intestinal segments, and muscular tissues. Herewith, *Clostridium perfringens* prevailed (17.5%). This bacterium isolates demonstrated multiple drug resistance to cefixime, fusidic acid, cefotaxime, cefaclor, spectinomycin, piperacillin, clarithromycin, doripenem and doxycycline.

Conclusion. The obtained results can be used for modification of current clostridial infection treatment protocols, reformulation of immunobiological products, development of evidence-based guidelines for use of antibiotics in livestock production to mitigate antimicrobial resistance risks.

Keywords: *Clostridium*, *Clostridiaceae*, cattle, antibiotic resistance, toxigenicity, biosafety, pathogenicity, anaerobes

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For correspondence: Pavel N. Shastin, Cand. Sci. (Veterinary Medicine), Senior Researcher, Laboratory for Diagnostics and Control of Antibiotic Resistance of Pathogens of the Most Clinically Significant Infectious Animal Diseases, Federal Scientific Centre VIEV, 24/1 Ryazansky prospekt, Moscow 109428, Russia, *shastin.pasha@yandex.ru*

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Видовое разнообразие клостридий у крупного рогатого скота

П. Н. Шастин, В. А. Савинов, А. И. Лаишевцев, Е. Д. Мандрыка, Е. А. Фабрикантова, А. В. Супова
ФГБНУ «Федеральный научный центр – Всероссийский научно-исследовательский институт экспериментальной ветеринарии имени К. И. Скрябина и Я. Р. Коваленко Российской академии наук» (ФГБНУ ФНЦ ВИЭВ РАН), Рязанский проспект, 24/1, г. Москва, 109428, Россия

РЕЗЮМЕ

Введение. Клостридиозы, несмотря на относительно спорадические случаи их возникновения, имеют повсеместное распространение и характеризуются высокой летальностью, что наносит экономический ущерб сельскому хозяйству. У крупного рогатого скота патогенные клостридии вызывают такие заболевания, как энтеротоксемия, злокачественный отек, столбняк, ботулизм. Этиологически значимыми видами клостридий являются *Clostridium septicum*, *Clostridium perfringens*, *Clostridium chauvoei*, *Clostridium novyi*, *Clostridium sordellii*.

Цель работы. Изучение видового разнообразия клостридий на основании исследований патолого-анатомического и секционного материала крупного рогатого скота из различных регионов России, определение мест их локализации в организме животных, а также антибактериальной устойчивости *Clostridium perfringens* к наиболее распространенным группам антибиотиков.

Материалы и методы. В период проведения исследования руководствовались общепринятыми нормативно-правовыми документами, методическими указаниями, рекомендациями, инструкциями; применяли микробиологические, масс-спектрометрические методы. Для определения антибактериальной устойчивости использовались различные группы препаратов: макролиды, монобактамы, пенициллины, полипептиды, гликопептиды, аминогликозиды, карбапенемы, линкозамиды, тетрациклины, ансамицины, диаминопиримидины, фузидины и др. Изоляты клостридий выделяли, используя рутинные бактериологические методы, видовую идентификацию выполняли с помощью времяпролетной масс-спектрометрии MALDI-ToF.

Результаты. При исследовании 359 образцов биоматериала было выделено и идентифицировано 137 изолятов клостридий (*Paraclostridium bifermentans*, *Clostridium perfringens*, *Clostridium tertium*, *Clostridium butyricum*, *Clostridium septicum*, *Clostridium sporogenes*, *Clostridium cadaveris*, *Clostridium sphenoides*, *Clostridium cochlearium*, *Clostridium sartagoforme*, *Clostridium chauvoei*, *Clostridium novyi*, *Clostridium sordellii*, *Clostridium paraputrificum*, *Clostridium* spp.), из которых 25 обладали патогенными и 17 – токсигенными свойствами. Чаще всего клостридии обнаруживали в печени, тонком и толстом отделах кишечника, мышцах. При этом выявлено превалирование *Clostridium perfringens* (17,5%). Установлена полирезистентность изолятов данного вида бактерии к цефиксиму, фузидиевой кислоте, цефотаксиму, цефаклору, спектиномицину, пиперациллину, кларитромицину, дорипенему, доксициклину.
Заключение. Полученные результаты могут быть использованы для модификации существующих протоколов лечения клостридиозов, корректировки состава иммунобиологических препаратов, разработки рекомендаций по использованию антибиотиков в животноводстве для снижения рисков развития антимикробной резистентности.

Ключевые слова: клостридии, *Clostridiaceae*, крупный рогатый скот, антибиотикорезистентность, токсигенность, биобезопасность, патогенность, анаэробы

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Для корреспонденции: Шастин Павел Николаевич, канд. вет. наук, старший научный сотрудник лаборатории диагностики и контроля антибиотикорезистентности возбудителей наиболее клинически значимых инфекционных болезней животных ФГБНУ ФНЦ ВИЭВ РАН, Рязанский проспект, 24/1, г. Москва, 109428, Россия, *shastin.pasha@yandex.ru*

INTRODUCTION

The genus *Clostridium* was first described by A. Prażmowski in 1880. Over 225 species of *Clostridia* have been currently identified in various regions of the planet. *Clostridia* are gram-positive rods that form spores. They are widespread in the environment, and are also part of the human and animal microbiome. However, only some of them are capable of causing diseases in animals [1, 2, 3]. Clostridial infections are characterized by high mortality. Due to the spore-forming ability of *Clostridia*, they can persist in the soil for a long time, thus posing a potential threat of the disease emergence [4, 5, 6]. The pathogen entry into the body of animals occurs mainly by ingestion of contaminated feed (alimentary route), through wounds or by inhalation. The main factors of *Clostridium* pathogenicity are exotoxins and enzymes [7, 8, 9, 10], which have hemolytic, necrotizing and lethal effects. The most potent toxins of clostridial origin are botulinum and tetanus neurotoxins, as well as epsilon toxin produced by *Clostridium perfringens* types B and D [11, 12, 13, 14].

The emergence of polyresistant *Clostridium* strains results in wider spread of clostridial infections. A number of scientists have noted low therapeutic efficacy of antibacterial drugs against the clinical manifestation of anaerobic enterotoxemia in young cattle, high mortality and the need for specific prevention [7, 15, 16, 17, 18, 19].

According to "Galen" component of the FGIS "VetIS", the list of registered vaccines against bovine clostridial infections in the Russian Federation is currently includes the following products: Clostrivax (Tecnovax S. A., Argentina); Coglavax (Ceva Sante Animale, France; Ceva-Phylaxia Veterinary Biologicals Company, Hungary); Clostbovac-8 (Vetbiochem LLC, Russia); Clostarm-9 (Armavir Biofactory, Russia); Cubolac (CZ Vaccines S. A. U., Spain); Antox 9 (Stavropol Biofactory, Russia); One Shot Ultra 8 (Zoetis Inc., USA); Scourguard 4KS (Zoetis Inc., USA).

The relevance and novelty of the work lies in obtaining data on the antibiotic resistance of the etiologically relevant *Clostridium* isolates, on the structure of the strains isolated from cattle, and on their toxigenic and pathogenic properties. The resulted data will contribute to the improvement of the clostridial infection control system in cattle, which in turn will reduce the economic losses in the livestock production.

The aim of the work was to conduct the monitoring studies to identify *Clostridia*, as well as to assess the level of antimicrobial resistance of *Clostridium perfringens* isolates recovered from cattle in various regions of Russia, and to study their toxigenic and pathogenic properties.

MATERIALS AND METHODS

The work was performed in 2022–2024 at the Laboratory for Diagnostics and Control of Antibiotic Resistance of Pathogens of the Most Clinically Significant Infectious Animal Diseases of the Federal Scientific Centre VIEV, as part of the state project (FGUG-2025-0003) supported by the Ministry of Science and Higher Education of the Russian Federation. As a result of our own research, monitoring data was obtained and the practical part was completed. Sectional and autopsy materials collected from cattle were delivered from various regions of Russia: Nizhny Novgorod, Moscow, Leningrad, Ryazan, Novosibirsk, Penza Oblasts and Republic of Mordovia.

Biological material. A total of 359 samples were examined (liver, heart, spleen, lung, kidney, muscle, small and large intestines, stomach, hoof sections, amniotic fluid, etc.).

Recovery of isolates, determination of their pathogenic and toxigenic properties. The study aimed at the recovery of the isolates of the microorganisms that are etiologically most relevant for commercial animal husbandry, namely the *Clostridiaceae* family, was implemented in accordance

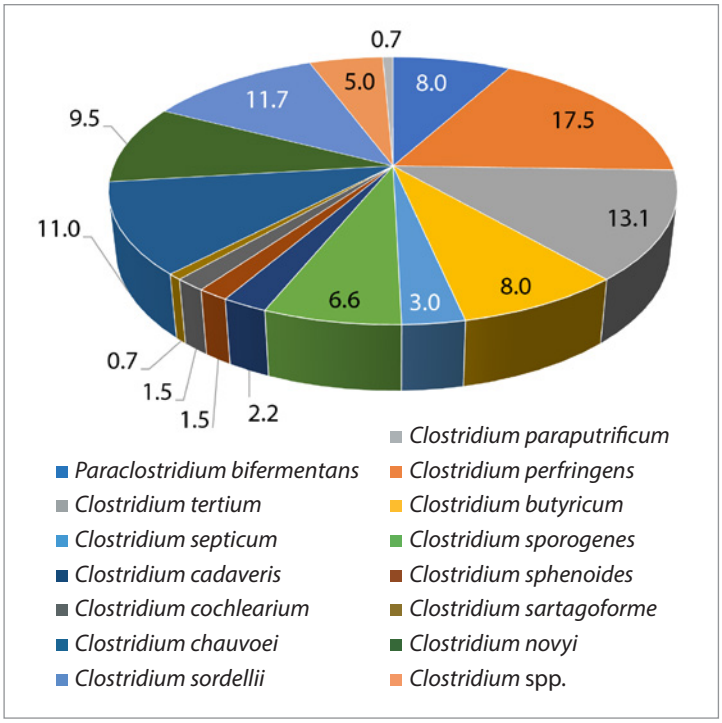

Fig. 1. Species diversity of Clostridium isolates circulating in the Russian Federation (n = 137), %

with GOST 26503-85 "Agricultural animals. Methods for laboratory diagnostics of clostridium"[1].

Identification of Clostridia. Species identification of the microorganisms was performed by mass spectrometry using MALDI Biotyper system (Bruker Daltonik GmbH, Germany) according to the "Guidelines for the identification of microorganisms using MALDI Biotyper mass spectrometer for the examination of food raw materials and food products" (approved by the Rosselkhoznadzor RTC on 3 April 2014).

Antibiotic resistance of the microbial cultures was determined by disc diffusion method in accordance with Methodological Guidelines MUK 4.2.1890-04 "Guidelines for susceptibility testing of microorganisms to antibacterial agents"[2]. Within the research activities, antibacterial drugs of various groups were used (HiMedia Laboratories Pvt Ltd., India): macrolides (azithromycin 15 µg, clarithromycin 15 µg, pristinamycin 15 µg, spiramycin 30 µg, tylosin 15 µg, erythromycin 15 µg), monobactams (aztreonam 30 µg), penicillins (amoxiclav 30 µg, amoxicillin 25 µg, ampicillin 25 µg, benzylpenicillin 10 µg, carbenicillin 100 µg, piperacillin 100 µg), polypeptides (bacitracin 10 µg, polymyxin B 50 µg), chloramphenicol 30 µg, glycopeptides (vancomycin 30 µg), aminoglycosides (gentamicin 30 µg, kanamycin 30 µg, spectinomycin 100 µg, streptomycin 25 µg), carbapenems (doripenem 10 µg), lincosamides (clindamycin 2 µg, lincomycin 10 µg), fluoroquinolones (levofloxacin 5 µg, norfloxacin 10 µg, ofloxacin 5 µg, pefloxacin 5 µg, ciprofloxacin 30 µg, enrofloxacin 10 µg), tetracyclines (oxytetracycline 30 µg, tetracycline 30 µg, chlortetracycline 30 µg, doxycycline 30 µg), ansamycins (rifampicin 15 µg), sulfonamides (sulfadiazine 100 µg, sulfafurazole 300 µg), diaminopyrimidines (trimethoprim 25 µg), cephalosporins (cefixime 5 µg, cefazolin 30 µg, cefaclor 30 µg, cefalexin 30 µg, cefotaxime 30 µg, cefepime 30 µg, cefoperazone 75 µg, cefpirome 30 µg, ceftriaxone 30 µg), phosphonic acid derivatives (fosphomycin 50 µg), fusidines (fusidic acid 30 µg).

The results were interpreted in accordance with CLSI (Clinical and Laboratory Standards Institute) and EUCAST (European Committee on Antimicrobial Susceptibility Testing) recommendations [20, 21].

The results were statistically processed using Microsoft Excel.

RESULTS AND DISCUSSION

As a result of the studies, 137 *Clostridium* isolates were recovered and identified: *Paraclostridium bifermentans*, *Clostridium perfringens*, *Clostridium tertium*, *Clostridium butyricum*, *Clostridium septicum*, *Clostridium sporogenes*, *Clostridium cadaveris*, *Clostridium sphenoides*, *Clostridium cochlearium*, *Clostridium sartagoforme*, *Clostridium chauvoei*, *Clostridium novyi*, *Clostridium sordellii*, *Clostridium paraputrificum*, *Clostridium* spp.

Diversity of *Clostridium* spp. circulating in the Russian Federation has been established, which is shown in Figure 1.

Prevalence of *C. perfringens* was established – 17.5%, followed by *C. tertium* – 13.1%, *C. sordellii* – 11.7%, *C. chauvoei* – 11.0%, *C. novyi* – 9.5%, *P. bifermentans* and *C. butyricum* – 8.0%, *C. sporogenes* – 6.6%, *Clostridium* spp. – 5.0%, *C. septicum* – 3.0%, *C. cadaveris* – 2.2%, *C. sphenoides* and *C. cochlearium* – 1.5% each, the smallest proportion is made up of *C. sartagoforme* and *C. paraputrificum* isolates – 0.7%.

Results of determination of antibiotic resistance of *C. perfringens* isolates ($n = 24$) recovered from cattle in various regions of the Russian Federation are demonstrated in Figure 2.

According to the obtained data, it can be concluded that *C. perfringens* isolates ($n = 24$) demonstrated resistance to cefixime, fusidic acid, cefotaxime, cefaclor, spectinomycin, piperacillin, clarithromycin, doripenem, and doxycycline. Antibiotic resistance to ampicillin demonstrated 85% of the isolates, to amoxicillin, chlortetracycline, vancomycin, rifampicin and ciprofloxacin – 80%, to tylosin and amoxiclav – 75%, to sulfadiazine, cefalexin, ofloxacin and polymyxin B – 60%, to pefloxacin and cefoperazone – 55%, to benzylpenicillin, clindamycin, ceftriaxone and chloramphenicol – 50%, to enrofloxacin, cefazolin, tetracycline and streptomycin – 45% of the isolates; 40% of *C. perfringens* isolates were resistant to bacitracin, norfloxacin, fosfomycin; 35% of the isolates demonstrated resistance to levofloxacin, lincomycin, oxytetracycline; 25% of isolates were resistant to erythromycin, spiramycin and gentamicin and 20% – to azithromycin, cefepime and cefpirome. All studied *C. perfringens* isolates were susceptible to sulfafurazole and carbenicillin (100%), trimethoprim – 90%, azithromycin – 70%, levofloxacin – 65%, and kanamycin – 45% of the isolates. All the tested strains were intermediately susceptible to aztreonam and pristinamycin, 75% of the isolates – to spiramycin, 60% – to fosfomycin, and 55% – to cefazolin, 45% – to kanamycin.

Among 137 recovered *Clostridium* isolates, 25 demonstrated pathogenic properties and 17 had toxigenic properties. The obtained data is presented graphically as a percentage in Figures 3 and 4.

[1] https://base.garant.ru/5916932 (in Russ.)

[2] https://docs.cntd.ru/document/1200038583 (in Russ.)

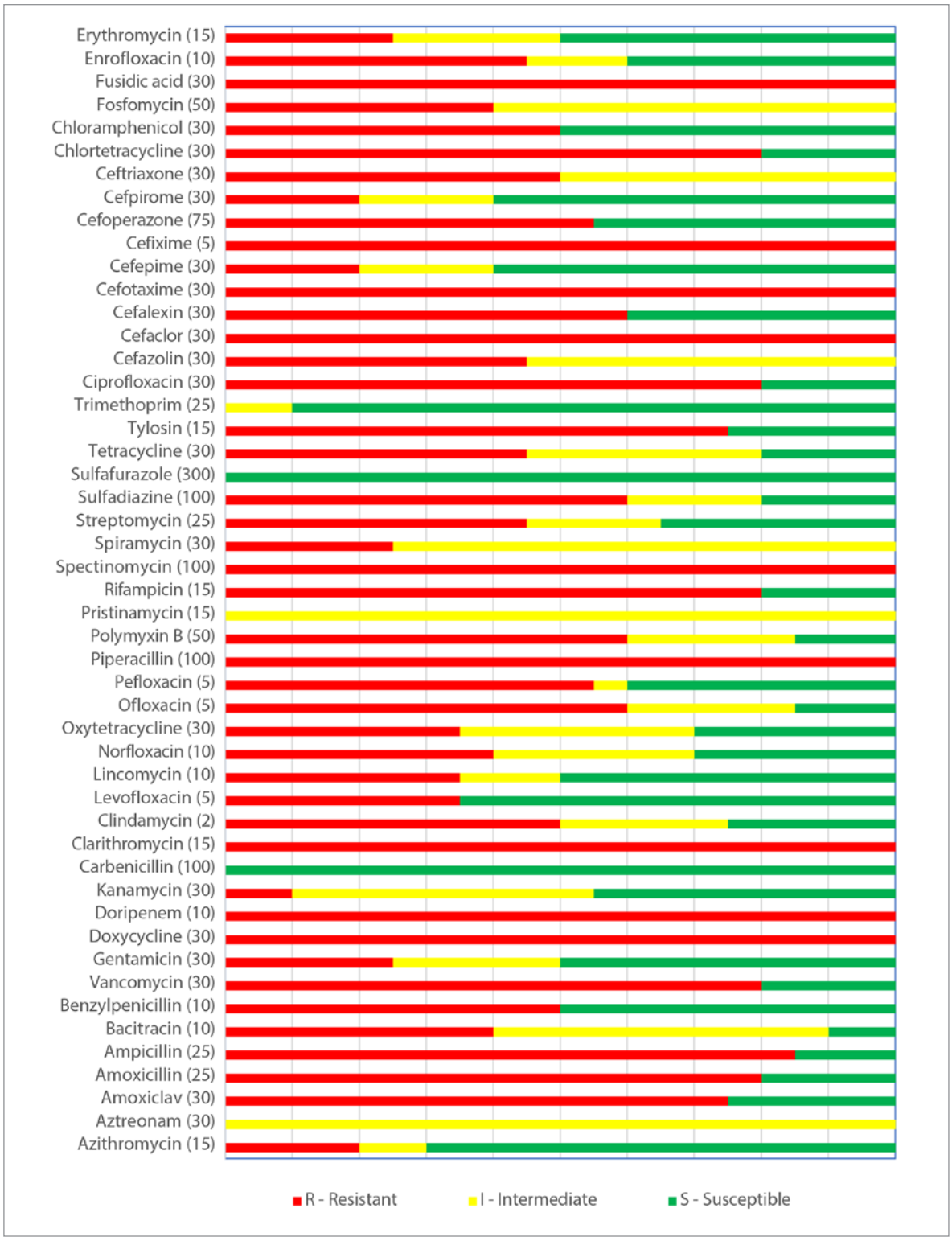

Fig. 2. Antibiotic resistance of C. perfringens isolates (n = 24) recovered from cattle

In most cases, *C. perfringens* isolates possessed pathogenic properties (6.6%). Pathogenicity factors were detected in 5.1% of *C. novyi* strains, 4.4% of *C. chauvoei* isolates, in 1.5% of *C. septicum* strains and in 0.7% of *Clostridium* spp. isolates. Toxigenic properties were determined for *C. sordellii* (3.7%), *C. perfringens* (3.7%), *C. novyi* (3.0%), *C. septicum* (1.5%) and *Clostridium* spp. (0.7%).

The localization sites of *Clostridia* in cattle are presented in the Table.

According to the demonstrated data, *Clostridia* were most often isolated from the liver, small and large intestine and from muscles.

Clostridia are widespread bacteria that cause diseases in animals, birds and humans. Antibiotic resistance is a serious challenge for the veterinary medicine due to the fact that 80% of all antibiotics in the world are used in agriculture, *inter alia* as feed additives and growth promoters. The results obtained during the present study

Table
Localization of *Clostridia* in cattle

Clostridium species	Biological material										
	Heart	Liver	Spleen	Lung	Kidney	Muscle	Small intestine	Large intestine	Stomach	Hoof sections	Amniotic fluid
Paraclostridium bifermentans	–	+	–	–	–	+	+	+	+	+	–
Clostridium tertium	–	+	+	–	–	+	+	+	–	–	–
Clostridium perfringens	–	+	+	–	+	+	+	+	+	–	+
Clostridium butyricum	–	+	–	–	–	–	+	+	+	–	–
Clostridium cochlearium	–	–	–	–	–	–	–	+	–	–	–
Clostridium sartagoforme	–	–	–	–	–	–	–	+	–	–	–
Clostridium septicum	–	+	–	–	+	+	–	+	–	–	–
Clostridium sporogenes	–	+	–	–	–	+	+	+	–	–	–
Clostridium sphenoides	–	–	–	–	–	–	–	+	–	–	–
Clostridium chauvoei	–	+	–	–	–	–	+	+	+	–	–
Clostridium novyi	–	+	–	–	–	–	–	–	–	–	–
Clostridium sordellii	–	+	–	–	–	–	–	–	–	–	–
Clostridium paraputrificum	–	+	–	–	–	–	–	–	–	–	–
Clostridium spp.	–	+	–	–	–	+	+	+	–	–	–
Clostridium cadaveris	–	+	–	–	+	–	–	+	–	–	–

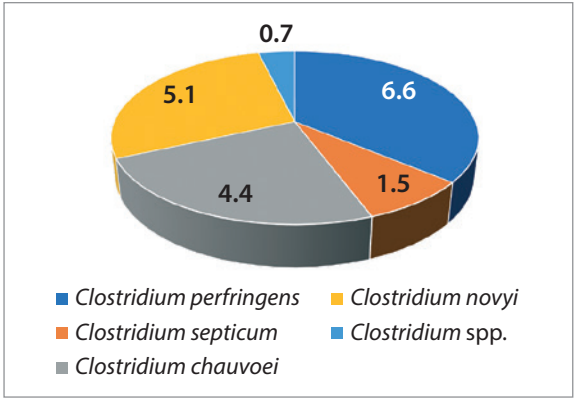

Fig. 3. Species composition of Clostridium isolates with pathogenic properties, %

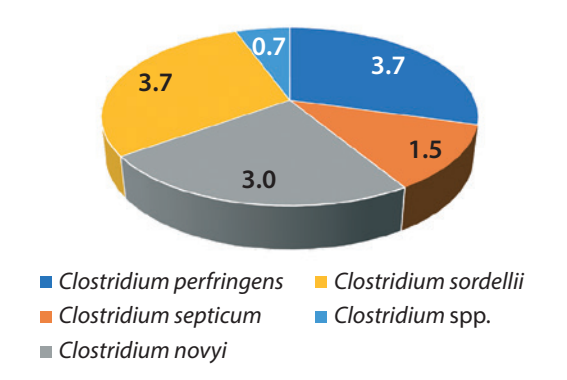

Fig. 4. Species composition of Clostridium isolates with toxic properties, %

on antibiotic resistance to cefotaxime are consistent with the data reported by N. A. Bezborodova et al. [7], H. A. Ahmed et al. [22]. In the studies carried out by the Iranian researchers F. Khademi et al., resistance of *C. perfringens* to ampicillin (25.8%), erythromycin (32.9%), gentamicin (45.4%), tetracycline (19.5%), amoxicillin (19.3%), bacitracin (89.1%) was reported [23]. A group of scientists from China and Pakistan studied eleven of the most commonly used antibiotics, two of them had no inhibitory effect, five were effective, and four had moderate effect against *C. perfringens*. Lincomycin and amikacin did not inhibit the isolates, tetracycline, penicillin, erythromycin and oxytetracycline inhibited *Clostridium* growth to a lesser extent. The scientists have concluded that it was advisable to use several types of antibiotics, which was a more effective approach to inhibit the bacterial infection [24]. Researchers from Ivory Coast determined in their studies that the level of antibiotic resistance of *C. perfringens* to tetracycline, doxycycline, chloramphenicol, and erythromycin ranged from 20 to 50% [25]. A group of scientists from South Korea, when studying the prevalence and resistance of *C. perfringens* to antibiotics, found that resistance to tetracycline was 100%, to ampicillin – 31.6%, to chloramphenicol – 68.4%, to metronidazole – 34.2% and to imipenem – 71%. The researchers also noted an important point of the combined resistance of 78.9% of the isolates to several antimicrobial drugs [26].

CONCLUSION

As a result of the examination of the sections and pathological materials from cattle in 2022–2024, 137 *Clostridium* isolates were recovered, of which 25 demonstrated pathogenic properties, and 17 – toxigenic ones. The most

common *Clostridium* localization sites included liver, large and small intestine, muscles, and stomach. The bacteria were also detected in kidneys, spleen, amniotic fluid, and hoof swabs.

Monitoring studies aimed at the determination of the antimicrobial resistance of *C. perfringens* isolates revealed their resistance to cefixime, fusidic acid, cefotaxime, cefaclor, spectinomycin, piperacillin, clarithromycin, doripenem, and doxycycline.

The results of this study can be used to modify existing treatment protocols for clostridial infections, adjust the composition of immunobiological products, and develop recommendations for the use of antibiotics in animal husbandry to reduce the risk of antimicrobial resistance developing.

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INFORMATION ABOUT THE AUTHORS / ИНФОРМАЦИЯ ОБ АВТОРАХ

Pavel N. Shastin, Cand. Sci. (Veterinary Medicine), Senior Researcher, Laboratory for Diagnostics and Control of Antibiotic Resistance of Pathogens of the Most Clinically Significant Infectious Animal Diseases, Federal Scientific Centre VIEV, Moscow, Russia; *https://orcid.org/0000-0001-7360-927X, shastin.pasha@yandex.ru*

Vasiliy A. Savinov, Cand. Sci. (Biology), Senior Researcher, Laboratories of Mycology and Antibiotics named after A. H. Sarkisov, Federal Scientific Centre VIEV, Moscow, Russia; *https://orcid.org/0000-0003-1891-0005, visik06@mail.ru*

Aleksey I. Laishevtsev, Cand. Sci. (Biology), Leading Researcher, Acting Head, Laboratory for Diagnostics and Control of Antibiotic Resistance of Pathogens of the Most Clinically Significant Infectious Animal Diseases, Federal Scientific Centre VIEV, Moscow, Russia; *https://orcid.org/0000-0002-5050-2274, a.laishevtsev@gmail.com*

Ekaterina D. Mandryka, Microbiologist, Laboratory for Diagnostics and Control of Antibiotic Resistance of Pathogens of the Most Clinically Significant Infectious Animal Diseases, Federal Scientific Centre VIEV, Moscow, Russia; *https://orcid.org/0009-0004-6941-9499, mandryka.ekaterina@yandex.ru*

Elizaveta A. Fabrikantova, Microbiologist, Laboratory for Diagnostics and Control of Antibiotic Resistance of Pathogens of the Most Clinically Significant Infectious Animal Diseases, Federal Scientific Centre VIEV, Moscow, Russia; *https://orcid.org/0000-0003-4465-8640, el@novokchenova.ru*

Anastasia V. Supova, Researcher, Laboratory for Diagnostics and Control of Antibiotic Resistance of Pathogens of the Most Clinically Significant Infectious Animal Diseases, Federal Scientific Centre VIEV, Moscow, Russia; *https://orcid.org/0000-0003-0728-538X, supova.nastya@yandex.ru*

Шастин Павел Николаевич, канд. вет. наук, старший научный сотрудник лаборатории диагностики и контроля антибиотикорезистентности возбудителей наиболее клинически значимых инфекционных болезней животных ФГБНУ ФНЦ ВИЭВ РАН, г. Москва, Россия; *https://orcid.org/0000-0001-7360-927X, shastin.pasha@yandex.ru*

Савинов Василий Александрович, канд. биол. наук, старший научный сотрудник лаборатории микологии и антибиотиков им. А. Х. Саркисова ФГБНУ ФНЦ ВИЭВ РАН, г. Москва, Россия; *https://orcid.org/0000-0003-1891-0005, visik06@mail.ru*

Лаишевцев Алексей Иванович, канд. биол. наук, ведущий научный сотрудник, и. о. заведующего лабораторией диагностики и контроля антибиотикорезистентности возбудителей наиболее клинически значимых инфекционных болезней животных ФГБНУ ФНЦ ВИЭВ РАН, г. Москва, Россия; *https://orcid.org/0000-0002-5050-2274, a.laishevtsev@gmail.com*

Мандрыка Екатерина Дмитриевна, микробиолог лаборатории диагностики и контроля антибиотикорезистентности возбудителей наиболее клинически значимых инфекционных болезней животных ФГБНУ ФНЦ ВИЭВ РАН, г. Москва, Россия; *https://orcid.org/0009-0004-6941-9499, mandryka.ekaterina@yandex.ru*

Фабрикантова Елизавета Алексеевна, микробиолог лаборатории диагностики и контроля антибиотикорезистентности возбудителей наиболее клинически значимых инфекционных болезней животных ФГБНУ ФНЦ ВИЭВ РАН, г. Москва, Россия; *https://orcid.org/0000-0003-4465-8640, el@novokchenova.ru*

Супова Анастасия Владимировна, научный сотрудник лаборатории диагностики и контроля антибиотикорезистентности возбудителей наиболее клинически значимых инфекционных болезней животных ФГБНУ ФНЦ ВИЭВ РАН, г. Москва, Россия; *https://orcid.org/0000-0003-0728-538X, supova.nastya@yandex.ru*

Contribution of the authors: Shastin P. N. – source selection and analysis, experimental and laboratory data analysis, paper writing; Savinov V. A. – source selection and analysis; Laishevtsev A. I. – paper editing, formal analysis, drawing conclusions; Mandryka E. D. and Fabrikantova E. A. – microbiological tests, data accumulation and systematization; Supova A. V. – microbiological tests, test result compilation and interpretation.

Вклад авторов: Шастин П. Н. – подбор и анализ источников по теме, анализ экспериментальных и лабораторных исследований, написание текста статьи; Савинов В. А. – подбор и анализ источников по теме; Лаишевцев А. И. – редактирование текста статьи, формальный анализ, формулировка выводов; Мандрыка Е. Д. и Фабрикантова Е. А. – проведение микробиологических исследований, сбор и систематизация данных; Супова А. В. – проведение микробиологических исследований, обобщение и интерпретация результатов исследования.