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MARCH | МАРТ VOL. 11 No. 1 2022

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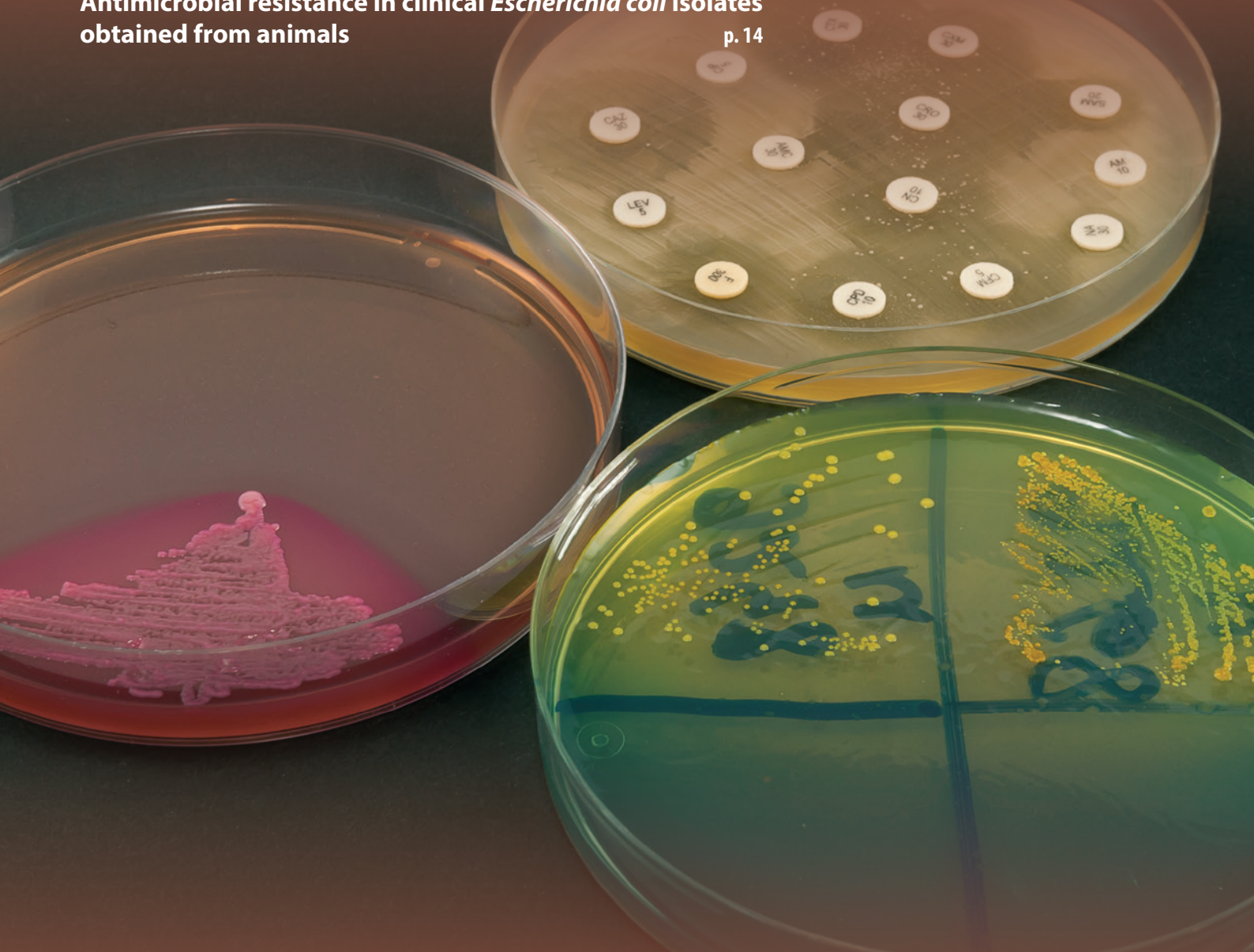

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Congratulatory massage of FGBI "ARRIAH" Director on 10th anniversary of Veterinary Science Today Journal

Dear readers! Ten years have passed since the foundation of the quarterly academic journal Veterinary Science Today by the FGBI "ARRIAH". We consider March 21, 2012 to be the starting point in its history. The journal was founded during the active phase of exotic disease introduction into our country. A new journal was needed that could become the main source of information on scientific achievements and their introduction into veterinary practice. Goals set by the first editorial board were achieved but they still remain relevant today. In the age of changing geopolitical reality the role of the journal should increase.

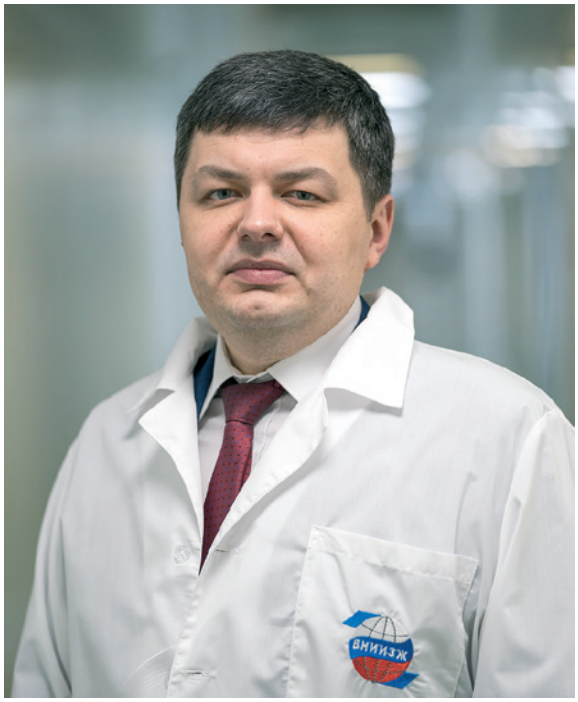
Over the ten years the journal grew into a serious periodical that publishes topical information on the issues dealing with all aspects of the development of the Russian and global veterinary science and practice.

From 2015, Veterinary Science Today Journal is in the List of Peer-Reviewed Scientific Publications designed to highlight key findings of Candidate of Science theses and Doctor of Science dissertations. Starting from the first issue in 2018, all papers in the journal are assigned a digital object identifier (DOI) and all publications are translated into English and entered into Crossref database. In 2019, International Standard Serial Number (ISSN) was assigned to the on-line edition of the journal and it is used to identify printed or digital serials irrespective of their language or carrier.

The scientific journal is included in the Directory of Open Access Journals (DOAJ) and in the list of journals included in the Russian Science Citation Index database powered by Web of Science as well as in the international database within EBSCOhost. Full-text electronic version of the journal is published on the Electronic Scientific Library web site – e-LIBRARY.RU.

Since the journal foundation, papers on innovative fundamental and applied research completed in general and veterinary virology, bacteriology, immunology, mycology, mycotoxicology, epizootology as well as reviews, peer-reviews and information messages about Russian and international research-to-practice events have been published in the journal.

Publication of the high-quality full-color journal is, indeed, hard and painstaking, but the editorial board successfully copes with its tasks!



Dear editorial board members! Please, accept my heartiest greetings to the anniversary and best wishes for success and further development for the good of Veterinary Medicine. May Veterinary Science Today Journal remain a reliable source of information, maintain its popularity and high authority among readers. Let you have new successful projects!

Best regards,
FGBI "ARRIAH" Director
Peter I. Kosyrev

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Current understanding of antimicrobial resistance mechanisms in bacteria (analytical review)

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SUMMARY
Data on mechanisms of resistance to antimicrobials in bacteria are reviewed and summarized. Main causes of resistance emergence and spread in bacteria are analyzed. Mechanisms of innate resistance of pathogenic bacteria (non-specific efflux pumps, antibiotic-inactivating enzymes and mechanisms serving as permeability barriers) are characterized. Mechanisms of acquired resistance are described: antibiotic modification or degradation; active removal of an antimicrobial from a bacterial cell – efflux (draining out); sequestration; target modification (bypass). The origin of antimicrobial resistance mechanisms in pathogenic bacteria is shown to be debatable. It is noted that producer microorganisms can directly transfer antimicrobial resistance genes to pathogenic bacteria, but a reliable link between this process and antimicrobial resistance spread has not been identified and proven so far. Horizontal gene transfer, including free DNA transformation, transduction by bacteriophages and plasmid-involving conjugation, is believed to play an important role in antimicrobial resistance spread. All three mechanisms are widespread in nature, although some bacterial species use one mechanism to a great extent than the other two. Transduction is supposed to play an important role, in particular, in the antibiotic resistance gene transfer, but the significance of transformation or transduction in the resistance gene transfer under the laboratory or environmental conditions has not been clarified so far due to the difficulty of naturally emerging recombination detection. Data on the role of conjugation in the antimicrobial resistance gene spread in nature, in particular carbapenem- and quinolone-resistance genes in Gram-negative and Gram-positive bacteria are presented. New trends in the antimicrobial resistance gene spread are indicated.

Keywords: review, antimicrobial resistance, antibiotics, mechanisms of antimicrobial resistance, bacteria, microorganisms

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Современное представление о механизмах антимикробной резистентности бактерий (аналитический обзор)

О. В. Прунтова[1], В. С. Русалеев[2], Н. Б. Шадрова[3]
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РЕЗЮМЕ
Проведен анализ и обобщены сведения о механизмах резистентности к антимикробным препаратам у бактерий. Рассмотрены основные причины возникновения и распространения устойчивости у бактерий. Охарактеризовано действие механизмов естественной резистентности патогенных бактерий (неспецифические эффлюксные насосы, инактивирующие антибиотики ферменты и механизмы, которые служат барьерами проницаемости). Описаны механизмы приобретенной устойчивости: модификация или разложение антибиотика; активное выведение антимикробного препарата из бактериальной клетки – эффлюкс (отток), секвестрация, модификация мишени (байпас). Показана дискуссионность вопроса о происхождении механизмов устойчивости к антибиотикам у патогенных бактерий. Отмечено, что прямая передача генов устойчивости к антимикробным препаратам может проис-

ходить от микроорганизмов-продуцентов к патогенным бактериям, но достоверная связь между этим процессом и распространением антимикробной резистентности в настоящее время не выявлена и не доказана. Роль горизонтальной передачи генов, включающей трансформацию свободной ДНК, трансдукцию бактериофагами и конъюгацию с участием плазмид, считают важной в распространении антимикробной резистентности. Все три механизма широко распространены в природе, хотя некоторые виды бактерий используют один механизм в большей степени, чем два других. Полагают, что трансдукция играет важную роль, в частности, в переносе генов устойчивости к антибиотикам, но до настоящего времени нет ясности в вопросе о значении трансформации или трансдукции в переносе генов резистентности в условиях лаборатории или в окружающей среде из-за сложности обнаружения рекомбинаций, возникших в естественных условиях. Представлены данные о роли коньюгации в распространении генов антимикробной резистентности в природе, в частности генов устойчивости к карбапенемам и хинолонам у грамотрицательных и грамположительных бактерий. Отмечены новые тенденции в распространении генов антимикробной резистентности.

Ключевые слова: обзор, антимикробная резистентность, антимикробный препарат, механизмы антимикробной резистентности, бактерии, микроорганизмы

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INTRODUCTION

Antimicrobial resistance (AMR), the resistance of bacteria to antimicrobials, is currently one of the most serious global problems. The long-term use of antibiotics to control animal and human disease agents resulted in that some bacteria have become resistant to drugs, and diseases have become non-responsive to treatment. According to the World Health Organization (WHO), already today many infections are caused by pathogenic microorganisms that are resistant to antimicrobials [1, 2].

The emergence and widespread of antibiotic-resistant forms of the bacteria that are non-susceptible to many antimicrobials are accompanied by a decrease in the therapy effectiveness, an increase in the treatment duration and in lethality. All this dictates the need to monitor animal bacteriosis agents, their structure and drug resistance level, and empirical antibiotic therapy of the disease, currently practiced by veterinarians, should take into account the actual data of epizootological monitoring of antibiotic resistance of the bacteria circulating in the particular livestock holdings.

This challenge has gone beyond the competence of the WHO and the World Organization for Animal Health (OIE) due to its complexity. Currently, it is recognized that no country or organization can alone cope with the AMR challenge [3, 4].

At first, the strategy for AMR prevention and containment including several directions shall be developed to address the AMR challenge. The key of which is the implementation of measures aimed at limiting and rational use of antimicrobials owing to the knowledge about antimicrobials resistance mechanisms in bacteria acquired by a wide range of veterinarians.

The Russian Federation participated in the drawing up of the Resolution on global strategy and global action plan for antimicrobial resistance adopted by the WHO Assembly in 2015. The said Resolution brought in force, urged all countries to carry out monitoring of drug-resistant bacterial infections and to ensure control of antimicrobials use in veterinary medicine, human medicine and agriculture as well as to strengthen international cooperation and funding in this field. In addition, international organizations have committed themselves to tighten legislative regulation of antimicrobials use, to search for their rational use (improvement of laboratory diagnostics of bacterioses, taking into account their susceptibility to antimicrobials) and to widely implement measures for infectious disease prevention, including vaccination, water purification, sanitary and hygiene measures [5].

In September 2017 the Government of the Russian Federation approved the 'Strategy for preventing of antimicrobial resistance in the Russian Federation for the period to 2030' [6] developed by the RF Ministry of Health. The Strategy lays down tasks for containment of the biological hazard associated with AMR spread and is aimed at prevention and limiting the resistance of microorganisms to antimicrobials.

Considering the significance of the above-said problem the paper is aimed at reviewing of national and foreign literature and description of mechanisms of antimicrobial resistance emergence and spread in bacteria.

MAIN CAUSES OF AMR EMERGENCE AND SPREAD IN BACTERIA

The phenotypic manifestation of AMR in bacteria is mediated by genetic properties, but not all and not always resistance genetic determinants manifest phenotypically. Resistance of bacteria to antimicrobials emerges and spreads due to the following:

– emergence of *random mutations* in genes capable of modifying activity spectrum of bacterial enzymes degrading antimicrobials;

– *exchange of genetic material between cells*, that is, the transfer of genes from resistant to less resistant or

susceptible microorganisms through the transfer of chromosomes, plasmids, phages, translocating elements;

– *selection of new resistant strains brought about by selective pressure of antimicrobials* associated with their uncontrolled use in various fields [7].

Traditionally, AMR mechanisms are considered only in relation to pathogenic microorganisms that have to protect themselves from the effects of medicinal products and disinfectants. And, accordingly, the main cause of AMR development is believed to be an anthropogenic impact on microorganisms. However, in the environment, antimicrobials- producing microorganisms that have to protect themselves from their metabolic byproducts are the primarily source of AMR genetic determinants rather than pathogenic microorganisms [8].

Antimicrobials-producing microorganisms, as a rule, have not one, but many complex self-protecting mechanisms that provide complete protection from the biologically active molecules that they produce. Moreover, some researchers have shown that self-resistance determinants are mostly linked to antimicrobials biosynthetic genes and their expression is co-regulated [9]. Therefore, natural reservoirs of resistance genes that may include the determinants conferring self-resistance to antimicrobials-producing microorganisms should be taken into account in addition to the often-mentioned AMR causes for full understanding of the antimicrobial resistance development in pathogenic microorganisms. Despite the fact that these resistance determinants in the environment microflora do not pose a threat to animal health, the transfer of these determinants to plasmids and integrons in pathogenic bacteria in the future may result in increase in the number of such determinants in pathogenic bacteria populations and the emergence of huge problems. That is, AMR spread prevention requires studies and control of the resistance determinant distribution in bacterial populations, clarification of the resistance mechanisms and determination of the environmental factors that contribute to their spread [8].

AMR MECHANISMS IN PATHOGENIC BACTERIA

As mentioned above, microorganisms have intrinsic and acquired AMR. Mechanisms of intrinsic resistance include nonspecific efflux pumps (that are supposed to emerge as a general response to environmental toxins), antibiotic-inactivating enzymes and mechanisms that serve as permeability barriers [10, 11]. These mechanisms are encoded by the main genetic structure – chromosome of bacterial cell. Well-studied efflux AcrAB-TolC pumping out system in *Escherichia coli* having broad substrate specificity and capable of outflow of antimicrobials and disinfectants of various classes is an example of intrinsic AMR [12]. Vancomycin-resistance in *E. coli* and Gram-negative bacteria is also a well-known example of intrinsic resistance emerging due to permeability barriers created by outer membrane [13]. Despite the fact that the intrinsic AMR mechanisms provide a low level of antimicrobials resistance, the normal commensal microflora of animals or environmental bacteria (water bodies, pastures) having intrinsic resistance mechanisms, can become opportunistic microorganisms in the animals with compromised immunity [14]. On the other hand, the mechanisms of acquired resistance in bacteria usually emerge as a result of horizontal gene transfer and also include specific efflux pumps encoded by a plasmid, for example, such as TetK and TetL in *Staphylococcus aureus*, as well as enzymes that can modify an antibiotic or an antibiotic target [15, 16]. These mechanisms pose a far more serious threat to human and animal health due to translocation of AMR determinants from chromosome to plasmid since it results in their enhanced expression and spread. Such an example is a transfer of the chromosomal AmpC *β*-lactamase gene into a plasmid, resulted in its worldwide spread [17].

MECHANISMS OF ACQUIRED ANTIMICROBIAL RESISTANCE IN PATHOGENIC BACTERIA

Biochemical mechanisms of AMR in pathogenic bacteria are very similar to the mechanisms found in producer microorganisms. Moreover, AMR genes in pathogenic bacteria belong to the same functional families as that ones of the producer microorganisms. AMR biochemical mechanisms are divided into several groups: antimicrobials modification or degradation; active antimicrobials removal from bacterial cell (efflux, outflow); sequestration of antimicrobials; target modification or bypass [18, 19].

MECHANISM OF ANTIMICROBIALS MODIFICATION OR DEGRADATION

This mechanism is commonly used by pathogenic bacteria to resist to aminoglycosides. The aim of antimicrobials modification is to render them ineffective, especially in the case of aminoglycoside antibiotics (for example, kanamycin, gentamycin and streptomycin), chloramphenicol and *β*-lactams. A large number of aminoglycoside-modifying enzymes including *N*-acetyl transferases, *O*-phosphotransferases and *O*-adenyltransferases that acetylate, phosphorylate or adenylylate aminoglycoside antibiotic are found in producer bacteria. These enzymes were first identified in early 1970s in members of *Streptomyces* species and then in other antibiotic-resistant pathogenic bacteria [20].

In pathogenic bacteria, genes coding for modification and degradation of antimicrobials are usually located on mobile genetic elements (MGE); chromosomal determinants have been also found in the majority of non-pathogenic environmental bacteria including those of *Providencia* and *Acinetobacter* genera [20]. These bacteria are considered a source of acquired AMR determinants found on MGEs in pathogenic strains. Of the known aminoglycoside-modifying enzymes, aminoglycoside-*N*-acetyltransferases are the most prevalent and well studied among pathogenic bacteria. Moreover, according to reports, some degradation enzymes were identified in both Gram-positive and Gram-negative bacteria [21]. However, *β*-lactamases are the modification/degradation enzymes most commonly used by pathogenic bacteria. While the role of *β*-lactamases in producer bacteria life is still debatable, they are known to play a critical role in *β*-lactam-resistance in Gram-negative bacteria. In Gram-positive bacteria, both penicillin-binding enzymes and *β*-lactamases play the main role in antimicrobials modification/degradation mechanism likely due to differences in their cell wall structures. More than 1,000 *β*-lactamases have been identified in pathogenic isolates of many bacteria species and their number continues to grow because of constantly emerging new mutations that allow them to adapt to new *β*-lactams. All currently known *β*-lactamases are classified into

four molecular classes based on common properties of enzymes and certain amino acid homology [22]. The majority of clinically significant β-lactamases belongs to Class A and Class C. In particular, Class A comprises β-lactamases of *Klebsiella* spp., *Citrobacter diversus*, *Proteus vulgaris* and the majority of *Bacteroides* spp. encoded by chromosome genes as well as almost all plasmid β-lactamases.

Class B enzymes are metallo-enzymes, since they contain a zinc atom as a coenzyme; they are prevalent in plasmids of *Enterobacteriaceae* family members. These enzymes are effective against penicillins, cephalosporins and carbapenems. *Beta*-lactamases of the following groups are significant in clinical practice: extended-spectrum β-lactamases of Gram-negative bacteria, cephalosporinases of Gram-negative bacteria, metallo-β-lactamases of Gram-negative bacteria [23]. TEM-3 β-lactamase can be taken as an example that is classified to extended-spectrum β-lactamases and is able to degrade the 3rd generation cephalosporins [24] that is indicative of rapid evolution of β-lactamases genes in pathogenic bacteria. Most β-lactamases are translocated to MGE facilitating their rapid spread in populations; however, some of β-lactamase genes can be present in chromosomes, for example, in members of *Enterobacteriaceae* family where they are poorly expressed and being silent genes. It can be supposed that, as in the case of aminoglycoside-modifying enzymes, β-lactamases may also perform dual functions including housekeeping and antibiotic resistance in bacteria [25]. Besides, biological function of β-lactamases in bacterial cell is supposed to be a remodeling peptoglycan cell wall but their gene translocation to plasmid results in high resistance to antimicrobials [17].

ACTIVE REMOVAL OF ANTIMICROBIALS FROM MICROBIAL CELL (EFFLUX, OUTFLOW)

Efflux is a commonly used mechanism of Gram-positive and Gram-negative bacteria to various antimicrobials such as β-lactams, fluoroquinolones, macrolides, lincosamides and tetracyclines. This mechanism uses different systems. The first one is *disorder of microbial cell membrane permeability*; this mechanism is common for Gram-negative bacteria having outer membrane and is less specific for antimicrobials of different groups. The second system is *decrease in permeability and/or antibiotic efflux from a bacterial cell*. Decreased permeability is important for Gram-negative bacteria because of the presence of the outer membrane that forms a permeability barrier and provides an intrinsic mechanism for protection from hydrophilic antibiotics, such as vancomycin [12]. Mutations in porin genes and/or changes in their expression were shown to have a further effect on Gram-negative bacteria susceptibility to hydrophilic antibiotics [26].

In addition, many types of active efflux pumps mediated by transport proteins were described in both Gram-positive and Gram-negative bacteria. Normally transport proteins carry out import or export of only one specific substrate. However, multi-drug or polyspecific exporters were found in natural microbial communities, suggesting that polyspecificity is widespread in natural microbial communities and is of ancient origin [27].

Genes encoding efflux pumps can be either intrinsic or acquired. Examples of intrinsic genes include AcrAB-TolC in *E. coli*, NorA in *St. aureus* and LmrA in *Lactococcus lactis*. Of these tripatite RND pump AcrAB-TolC is the most studied system. Although this system carries out efflux of very broad spectrum of compounds, its biological function is believed to be export of bile salts in *Enterobacteriaceae* family members [28]. The acquired antimicrobials efflux determinants often found on MGEs in pathogenic bacteria are represented by many different types of Tet genes (at least 22 genes have been identified) located on plasmids in both Gram-negative and Gram-positive bacteria [29].

SEQUESTRATION OF ANTIMICROBIALS

Sequestration involves proteins that bind to antimicrobials and prevent them from reaching their targets. This mechanism is more typical for producer microorganisms, for example, bleomycin producers – members of *Streptoalloteichus hindustanus*, *Streptomyces verticillus* and *Streptomyces flavoviridis* species which primary mechanism of resistance involves sequestration of the metal-bound or metal-free antibiotic [30].

TARGET MODIFICATION/BYPASS

This mechanism involves generation of additional targets or subunits in antimicrobials that that prevent them from binding, for example, methylation [18, 19]. Target modification acts as a self-resistance mechanism against several classes of antibiotics including β-lactams, glycopeptides, macrolides, lincosamides, streptogramins and aminoglycosides. A large number of such mechanisms were found in pathogenic bacteria. Methicillin-resistant *St. aureus* strains where β-lactam resistance is mediated by exogenous penicillin-binding protein which transpeptidase domain is not susceptible to several different β-lactams is a classical example of target modification. For example, β-lactam antibiotic has a similar structure to the substrates-peptidoglycan precursors that allow the antibiotic to associate and cause acetylation of active site serin resulting in its inhibition [31]. Resistance to vancomycin resulting from acquisition of Van gene cluster and being a typical cause of AMR in enterococci is another example of target modification [32]. In particular, VanA *и* VanB genes out of many known genes in this cluster determine AMR in pathogenic bacteria since they are found on MGEs [33].

Other examples of the target modification in pathogenic bacteria include point mutations or enzymatic alterations of the target [34]. Enzymatic alteration of the target is best understood in the case of macrolide resistance conferred by a large group of erythromycin ribosomal methylation (Erm) genes. These enzymes methylate a specific adenine in the 23S rRNA [35]. In pathogenic bacteria, Erm genes are present on MGEs and are widespread in both Gram-positive and Gram-negative bacteria [35, 36]. The most known examples of target protection in pathogenic bacteria include Tet(M) and Tet(O) proteins encoded by genes located on MGEs in *St. aureus*. These proteins were shown to be homologous to elongation factors, EF-G and EF-Tu, and their binding to ribosome facilitates removal of tetracycline from a bacterial cell in GTP-ase activity-dependent manner [37]. Thus, it can be concluded that that the most AMR mechanisms in bacteria appear to emerge from intercellular mechanisms of resistance to environmental conditions and it is the incorporation of AMR genetic determinants in MGEs in pathogenic bacteria that poses a serious threat to animal and human health.

THE ORIGIN OF ANTIMICROBIAL RESISTANCE IN PATHOGENIC BACTERIA

The question of how genes of resistance to antimicrobials emerge in pathogenic bacteria remains debatable. The idea that pathogen resistance genes could be obtained from antimicrobials –producer microorganisms by horizontal transfer was first proposed in the 1970s [38]. Despite sound evidence that their transfer from producer organisms to pathogenic strain might occur, a direct link between producers and pathogens has not been established and demonstrated so far. This is primarily due to the fact that resistance genes in producers demonstrate high sequence divergence and very different G + C content as compared to determinants in pathogens even when they use similar mechanisms. However, evolutionary link between determinants and pathogens is not denied [39]. Analysis of the data available in literature suggests that transfer of these determinants from producers to pathogenic bacteria could occur through a series of closely related non-producing actinomycetes in soil and then, finally, to proteobacteria and distant (non-related) pathogenic species [18].

ROLE OF HORIZONTAL GENE TRANSFER (HGT) IN BACTERIA AMR

Transfer of AMR genetic determinants between bacterial populations occurs by the mechanism including transformation with free DNA, transduction by bacteriophages or conjunction involving plasmids [14], collectively referred to as HGT mechanisms. All three HGT mechanisms are widely used in nature, although certain species of bacteria employ one mechanism more heavily than the other two [40]. Streptococci, for example, employ transformation whereas enterococci employ conjugative plasmids for exchange of genetic information. Transformation is best characterized in Gram-positive *Streptococcus pneumoniae* and *Bacillus subtilis*, although it also occurs in many Gram-negative bacteria. Although the role of transformation in bacteria physiology is still debated, its main purpose is believed to be DNA repair or genetic diversification for bacteria adaptation enhancement [41]. Indeed, transformation seems to have played important role in evolution of antibiotic-resistant members of *Streptococcus* and *Neisseria* genera. It is commonly thought that transduction also play a major role in AMR evolution in *St. aureus*, although it has been shown to occur in many bacteria at a low frequency ranging between 10^{-6} and 10^{-9} transductants/plaque-forming unit [42]. *St. aureus* species members are highly variable bacteria and have a large accessory genome consisting of phages, plasmids, transposons, genomic islands, etc. Traditionally, it is believed that HGT in general and transduction in particular play a major role in antimicrobials-resistance gene transfer [43], but due to the difficulty of recombinant event detection in natural conditions (outside the laboratory) the contribution of transformation or transduction to the AMR genes transfer in the clinic or in the environment remains unclear. Plasmid-mediated conjugation is still considered more important mechanism for AMR gene dissemination in nature than transformation or transduction because plasmids are capable of autonomous transfer both in the environment and in the laboratory [44]. This is confirmed by the most well known plasmids that have resulted in dissemination of carbapenem- and quinolone-resistance genes in Gram-negative bacteria over the very long geographical distances [45]. Other DNA elements in Gram-positive bacteria known as conjugative transposons or integrative conjugative elements can also mediate conjugation. These elements can both integrate into and excise from the chromosome and transfer themselves to other bacteria through conjugation [46]. Resistance gene transfer by conjugation requires high-density bacteria settings such as the human and animal gut, biofilms in the environment, animal keeping facilities [45, 46]. According to the generally accepted concept, some resistance determinants have been plasmid-associated for a long time, while other are mobilized to plasmids from chromosomes and rate of such gene mobilization has increased for the last 70 years, that is accounted for the widespread use of antibiotics [47].

New trends for AMR gene dissemination are as follows:

– increase in rate of resistance determinant mobilization from chromosomes to plasmids;

– clustering of antimicrobials-resistance genes in plasmids probably in response to selective pressure in the environment. A well-characterized mechanism of clustering is demonstrated by *St. aureus* pSK41 conjugative plasmid containing an insertion sequence IS 257 that promotes capture of small resistance plasmids [43].

CONCLUSION

Use of antimicrobials as medicines, disinfectants and feed additives in various industries is considered to be one of the major causes of challenges associated with AMR emergence and spread in bacteria. Natural sources of AMR are usually not taken into account.

Analysis of domestic and foreign literature allows us to conclude that:

1. Natural antimicrobials-producing microorganisms have been and continue to be the primary sources of AMR genetic determinants. Despite of barriers to the exchange of genetic information between different genera of bacteria, widespread transfer of AMR genes from chromosomes of environmental bacteria to mobilized elements of pathogenic bacteria has occurred.

2. Antimicrobials – producing microorganisms, as a rule, have not one, but many complex self-defense mechanisms conferring complete protection from biologically active molecules produced by them, and the genetic determinants of self-resistance are almost always clustered with the antimicrobials biosynthesis genes and their expression is co-regulated. Therefore, to better understand AMR evolution, natural reservoirs of resistance genes should be considered in addition to frequently mentioned AMR causes that may comprise self-resistance determinants of antimicrobials-producing microorganisms since such resistance determinants in environmental microflora could result in further increase in number of such determinants in pathogenic bacteria populations and tremendous challenges. That is, studies and control of AMR determinant distribution in bacterial populations, clarification of resistance mechanisms identification of environmental factors that contribute to their spread are required to prevent AMR spread [8].

3. The majority of AMR mechanisms in bacteria appeared to emerge from intercellular mechanisms of resistance to the environment and the inclusion of AMR genetic determinants in the MGE of pathogenic bacteria poses a serious threat to animal and human health. Based

on the analysis of data in the available literature, it can be assumed that the transfer of these determinants from producers to pathogenic bacteria could occur through a number of indelibly linked closely related non-producing actinomycetes in the soil and only then to proteobacteria and distant (non-related) pathogenic bacteria species.

4. New trends for AMR gene dissemination are as follows: increase in rate of resistance determinant mobilization from chromosomes to plasmids observed during the last 70 years and clustering of antibiotic-resistance genes in plasmids probably in response to selective pressure in the environment. A well-characterized mechanism of clustering is demonstrated by *St. aureus* pSK41 conjugative plasmid containing an insertion sequence IS 257 that promotes capture of small resistance plasmids.

Prompt detection of changes in antimicrobials resistance dissemination in bacteria is of great practical and theoretical importance, as it allows update of the recommendations for antibacterial therapy in animal farming industry, development of express molecular methods for antimicrobial resistance detection and provides important information for the creation of new medicinal products that can overcome the resistance.

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Antimicrobial resistance in clinical *Escherichia coli* isolates obtained from animals

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SUMMARY
The article presents data on the phenotypic and genotypic characteristics of antimicriobial resistance in *Escherichia coli* clinical isolates recovered from bovine microbiota (secretions from mammary glands, cervical swabs). 127 *Escherichia coli* isolates were studied, i.e. 44 from mammary glands secretions and 83 from cervical swabs. Disk diffusion method was used to study antimicrobial resistance of the cultures; minimum inhibitory concentrations of antimicrobials were determined in a serial dilution method; resistance genes were detected by polymerase chain reaction. The carried out research demonstrates a wide distribution of the isolates belonging to the phenotype resistant to ansamycins (rifampicin), semi-synthetic penicillins (ampicillin and amoxicillin), tetracyclines (doxycycline). The isolates showed a lower level of resistance to macrolides (azithromycin), amphenicols (levomycetin) and aminoglycosides (tobramycin). It was found that *Escherichia coli* clinical isolates are sensitive to third-generation cephalosporins and fluoroquinolone antimicrobials. However, since 28.46% of cultures demonstrate intermediate resistance to third-generation cephalosporins and 49.02% of *Escherichia coli* DNA samples isolated from mammal gland secretions had blaDHA gene associated with resistance to this group of antimicrobials, these antimicrobials could be hardly recommended as antibiotics of choice. Absence of VIM carbapenemase-encoding gene in the DNA of the recovered isolates and a low level of phenotypic resistance (10.22% of isolates from cervical swabs) can be one of the reasons for recommending first-line carbapenems as antibiotics of choice to treat animal diseases associated with *Escherichia coli*, along with fluoroquinolones as reserve antimicrobials. It was found that the recovered *Escherichia coli* isolates are more sensitive to combination antibiotics than to mono-antibiotics.

Keywords: phenotypic and genetic resistance, *Escherichia coli*, isolates, genetic markers, microbiota, Gram-negative bacteria, extended-spectrum β-lactamases, antibiotics

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РЕЗЮМЕ

Представлены данные о фенотипической и генотипической характеристике антибиотикорезистентности клинических изолятов *Escherichia coli*, выделенных из микробных биотопов (секрет молочной железы, цервикальные смывы) крупного рогатого скота. Исследовано 127 изолятов кишечной палочки, в том числе 44 – из секрета молочной железы, 83 – из цервикальных смывов. Антибиотикорезистентность культур изучали диско-диффузионным методом, минимальные ингибирующие концентрации антибактериальных препаратов определяли методом серийных разведений, гены резистентности детектировали с помощью полимеразной цепной реакции. В результате исследований показано широкое распространение изолятов микроорганизмов с фенотипом резистентности к ансамицинам (рифампицину), полусинтетическим пенициллинам (ампициллину и амоксициллину), тетрациклинам (доксициклину). Меньший уровень устойчивости изоляты проявляли к макролидам (азитромицину), амфениколам (левомицетину) и аминогликозидам (тобрамицину). Установлено, что клинические изоляты *Escherichia coli* чувствительны к цефалоспоринам III поколения и противомикробным средствам из группы фторхинолонов. Однако регистрация у 28,46% культур промежуточной резистентности к цефалоспоринам III поколения и выявление гена blaDHA, ассоциированного с развитием устойчивости к данной группе препаратов в 49,02% образцов ДНК эшерихий, изолированных из секрета молочной железы, не позволяют рекомендовать их в качестве препаратов выбора. Отсутствие гена VIM, кодирующего продукцию карбапенемаз в ДНК у выделенных изолятов, и низкий уровень фенотипической устойчивости (10,22% изолятов из цервикальных смывов) может служить одной из предпосылок для рекомендации использования карбапенемов 1-го ряда в качестве препаратов выбора для терапии заболеваний животных, ассоциированных с *Escherichia coli*, наряду со фторхинолонами, однако только в качестве препаратов резерва. Установлено, что выделенные изоляты *Escherichia coli* демонстрировали бóльшую чувствительность к комбинированным противомикробным лекарственным средствам в сравнении с монопрепаратами.

Ключевые слова: фенотипическая и генетическая резистентность, *Escherichia coli*, изоляты, генетические маркеры, микробные биотопы, грамотрицательные бактерии, *β*-лактамазы расширенного спектра, антибиотики

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INTRODUCTION

Escherichia coli is the most widespread Gram-negative bacterial pathogen, that poses both clinical and epidemiological problems resulting in a number of infectious and inflammatory diseases in animals [1–3].

Violation of antimicrobial chemotherapy schemes and protocols observed now in livestock production causes occurrence of *E. coli* resistant strains in animals. Those strains can be transmitted to humans, inter alia, through objects and food products contaminated with bacteria resistant to antibiotics, which is a serious epidemiological threat [3–7].

Antimicrobial resistance of *E. coli* isolates is explained both by the natural resistance of the microorganism to the main clinically significant antimicrobial substances and by the genetically determined molecular mechanisms of resistance and virulence, mostly developed due to the horizontal transfer of a gene encoding them [8–11]. Extrachromosomal factors form the basis for rapidly emerging resistance, which can be developed within 1–2 years [12].

Monitoring data on antimicrobial resistance (AMR) of *E. coli* clinical isolates from animals in the Russian Federation are incomplete and differ depending on the region and on the study period [13–15].

At the same time, in order to ensure effective antimicrobial therapy for farm animals, it is necessary to look into *E. coli* resistance to antimicrobials.

In connection with the above, the aim of this work was to study phenotypic and genotypic characteristics of antimicrobial resistance in *E. coli* clinical isolates recovered from cattle in the Ural region.

MATERIALS AND METHODS

The tested samples. From 2016 to 2021, *E. coli* isolates (n = 127) recovered from bovine clinical materials were received from different livestock farms of the Ural region.

Nutrient media or reagents. The following differential diagnostic nutrient media were used in the work: Endo medium, Levin medium, sorbitol medium, meat-peptone agar (MPA), blood agar, Manka agar, Olkenitsky's medium, Simmons medium (FBIS SRCAMB, Russia). Biochemical properties of bacterial cultures were determined with a set of reagents "Biochemical assay plates for enterobacteria differentiation (PBDE)" (RPC Diagnostic Systems, Russia) according to the manufacturer's instructions.

Microbiological tests were carried out in accordance with the "Methodical guidance on bacteriological diagnosis of colibacillosis in animals", approved by the Ministry of Agriculture of the Russian Federation on July 27, 2000 No. 13-7-2/2117 [16].

The following biochemical properties of isolated *E. coli* cultures were studied: production of urease, *β*-D-galactosidase, *β*-glucosidase, phosphatase, lysine decarboxylase,

ornithine decarboxylase, arginine dihydrolase, nitrite reductase, hydrogen sulfide, indole, acetoin (acetylmethylcarbinol); fermentation of glucose, sucrose, mannitol, trehalose, lactose, mannoses, xyloses, riboses, cellobioses, malonate, citrate, sodium citrate with glucose, inositol, sorbitol, arabinose, maltose.

The results of biochemical reactions were assessed visually. The isolated cultures were identified in accordance with Bergey's Manual of Determinative Bacteriology[1].

The resistance phenotype observed in 15 recovered isolates to the following antimicrobials (included into 10 groups: ansamycin, tetracyclines, third-generation cephalosporins, penicillins, macrolides, second generation fluoroquinolones, amphenicols, aminoglycosides, glycopeptides, carbapenems) was determined in a disk diffusion test in accordance with MUK 4.2.1890-04 "Determination of sensitivity of microorganisms to antimicrobials" [17]. For the purposes of this work we used standard commercial disks (LLC "NITSF", Russia) with the known active ingredient content: meropenem – 10 μg, ciprofloxacin – 5 μg, rifampicin – 5 μg, ofloxacin – 5 μg, ampicillin – 10 μg, amoxicillin – 20 μg, levomycetin – 30 μg, doxycycline – 30 μg, ceftriaxone – 30 μg, enrofloxacin – 5 μg, tetracycline – 30 μg, azithromycin – 15 μg, vancomycin – 30 μg, gentamicin – 120 μg, tobramycin – 10 μg. The results were interpreted in accordance with the recommendations of the European Committee on Antimicrobial Susceptibility Testing [18].

Minimum inhibitory concentrations (MIC) of antimicrobials, including combined pharmaceuticals intended for treatment of inflammatory diseases of the mammary gland and organs of reproductive tract (for isolates recovered from mammary gland secretions and cervical swabs of animals), was determined in a serial dilution method with addition of a bacterial suspension containing 10^7 bacteria in 1 mL. Broth and a suspension of bacteria without an antibiotic were used to control the purity of the growth of cultures, and broth with an antibiotic without culture was used to control the sterility of the medium. The inoculations were incubated in a thermostat for 24 hours [17].

Molecular genetic tests using polymerase chain reaction (PCR) were carried out in accordance with the instructions for commercial test systems. We used a DNA isolation kit Diatom DNA Prep 200 for our work (Isogen Laboratory LLC, Russia). Genetic determinants of AMR resistance were detected in PCR using reagent kits from "Lytech" Co. Ltd. (Russia). We detected blaDHA gene encoding plasmid-mediated *β*-lactamase AmpC and causing resistance of *Enterobacteriaceae* bacteria to protected penicillins and broad-spectrum cephalosporins [16, 17, 19, 20]; CTX-M gene encoding extended-spectrum *β*-lactamases, which is moved by mobile genetic elements (transposons, integrons, IS elements) and is associated with development of multi-resistance; VIM gene located on a non-conjugative plasmid, which includes class 1 integron and causes production of carbapenemases. Real-time PCR was performed using analyzer from Applied Biosystems QuantStudio™ 5 (Thermo Fisher Scientific Inc., USA).

[1] Bergey's Manual of Determinative Bacteriology: two volumes. Vol. 1. Ed. J. G. Holt, N. Krieg, P. Snit, J. Staley, S. Williams. 9th ed. Moscow: Mir; 1997. 432 p.

For statistical data analysis we used a standard Microsoft Excel 2010 package and methods of descriptive statistics: percentages, frequencies, frequency distribution, etc.

RESULTS AND DISCUSSION

Detecting AMR E. coli isolates with a disk-diffusion method. The figure below shows results of AMR study for 127 *E. coli* isolates recovered from bovine biological materials.

The AMR profile of *E. coli* isolates recovered from mammary glands secretions was characterized by high resistance levels (54.54%) to the penicillin group, i.e. 49.99% of cultures were resistant to ampicillin, and 4.55% to amoxicillin. Resistance to rifampicin and the tetracycline group was observed in 47.72% and 45.46% of *E. coli* isolates, respectively. 15.91% of *E. coli* cultures were resistant to amphenicol group (levomycetin). Resistance to aminoglycosides (tobramycin) was detected in 11.36% of isolates. The minimum number of AMR *E. coli* isolates was found in relation to the following groups of antimicrobials: second-generation fluoroquinolones (ciprofloxacin – 2.27%, enrofloxacin – 4.55%, ofloxacin – 6.82%), macrolides (azithromycin – 4.55%), third generation cephalosporins (ceftriaxone – 6.82%).

It was found that 31.81% of *E. coli* isolates recovered from secretion of the bovine mammary glands showed intermediate resistance to tobramycin, which belongs to the group of aminoglycosides. 29.55% of isolated *E. coli* cultures demonstrated intermediate resistance to levomycetin, 22.73% of *E. coli* isolates demonstrated resistance to ciprofloxacin and ceftriaxone. Intermediate resistance to representatives of tetracycline (doxycycline) group and second-generation fluoroquinolone group (enrofloxacin) was reported in 15.91% of isolates.

As for *E. coli* isolates from bovine cervical swabs, phenotypes resistant to penicillin antimicrobials were predominant: 42.17 and 36.15% of cultures were resistant to ampicillin and amoxicillin. 53.01% of isolates demonstrated resistance to rifampicin, which is the main representative of the ansamycin group. 25.30% of *E. coli* isolates were resistant to doxycycline, and 7.23% were resistant to tetracycline. The minimum number of *E. coli* isolates was resistant to third-generation cephalosporins (3.61%) and aminoglycosides, namely gentamicin (2.41%). 32.53% of isolates demonstrated intermediate resistance to ceftriaxone, which is a representative of the third-generation cephalosporins. Intermediate resistance to the carbapenem group was revealed in 30.65% of isolates. 27.02% of *E. coli* cultures had intermediate resistance to levomycetin. Intermediate resistance to representatives of second-generation fluoroquinolones, namely ciprofloxacin, ofloxacin and enrofloxacin, was demonstrated by 16.87, 22.8, and 16.87% of isolates, respectively.

AMR tests of 127 isolates of *E. coli* estimated that many of them had polyresistance: 64.5% of the studied cultures were resistant to four AMR classes, 54.33% – to five classes. 16.6% of bacterial isolates were multi-resistant, i.e. demonstrated resistance to six AMR classes.

Determination of minimum inhibitory concentrations (MIC) of commercial antimicrobials. *E. coli* isolates obtained from the secretion of the mammary gland (13.64%) demonstrated the highest rate of resistance to medicines, which include cloxacillin (penicillin group) as

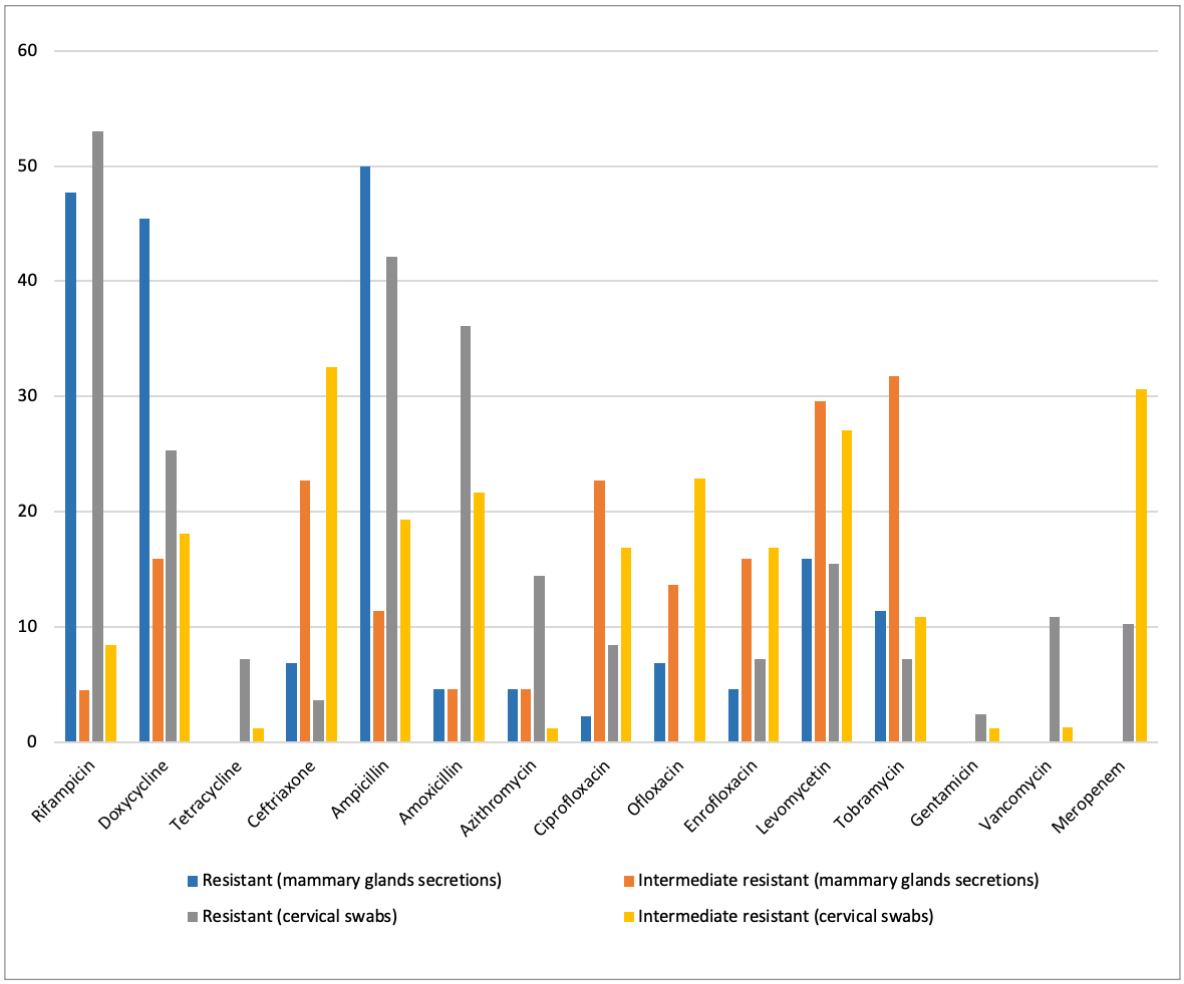

Fig. AMR profile of E. coli clinical isolates recovered from cattle (%)

the active substance. Cloxacillin-based combination of antibiotics were most active against *E. coli*. So, the combined use of such antimicrobials as cloxacillin + benzathine and cloxacillin + neomycin, sulfate + dexamethasone + trypsin caused resistance in only 6.82% of isolates, and combination of cloxacillin + ampicillin + benzathine acid caused resistance in 2.27% of isolates. 6.81% of *E. coli* cultures demonstrated resistance to antimicrobials belonging to the first-generation cephalosporins based on cefalonium and cefapirin. The least rate of resistance was observed to combination of antibiotics from tetracyclines (2.27%) and aminoglycosides (1.30%) groups. 4.34 and 4.55% of isolates showed intermediate resistance to second and third generation cephalosporin antimicrobials, respectively.

E. coli cultures isolated from cervical swabs of cows demonstrated the highest level of resistance to antimicrobials based on a synthetic antifungal agent from the imidazole derivatives – 12.05%. 6.02% and 8.40% of isolates were resistant to commercial antimicrobials containing substances from macrolide and ansamycin groups, respectively. 4.82% of isolates had intermediate resistance to a polypeptide antibiotic based on colistin sulfate and tylosin tartrate. 4.80% of isolates demonstrated intermediate resistance to the antimicrobial based on chlortetracycline hydrochloride.

Detecting genetic determinants of E. coli resistance. Molecular and genetic studies revealed that 9.56% of *E. coli* isolates have CTX-M gene in specific regions of DNA, which causes resistance of *Enterobacteriaceae* bacteria to first-generation fluoroquinolones and cephalosporins (cefazolin), in this case 6.95% of cultures were isolated from mammary glands secretions and 2.61% were isolated from cervical swabs. blaDHA gene, which accounts for resistance to protected penicillins (ampicillin, amoxicillin, ticarcillin, piperacillin, tazobactam) and the third- and the forth- generation cephalosporins (cefotaxime, cefoperazone, ceftriaxone, ceftibuten, ceftazidime, cefixime, cefpodoxime, cefodizime, cefetamet), had been identified in 49.02% of *E. coli* DNA samples isolated from mammary gland secretions. VIM gene encoding production of carbapenemases and accounting for resistance to the 1st line carbapenems (meropenem, imipenem, doripenem) was not detected in any of the *E. coli* DNA samples.

CONCLUSION

Analysis of the phenotypic and genotypic characteristics of 127 *E. coli* clinical isolates showed that microorganism cultures (64.5%) resistant to ansamycins, semi-synthetic penicillins, tetracyclines (doxycycline) prevail.

Fluoroquinolones were most active against *E. coli* and therefore can be recommended to treat reproductive

diseases in cows caused by this pathogen. Clinical isolates of *E. coli* were sensitive to third generation cephalosporins. However, since 28.46% of isolates in this group had shown intermediate resistance and due to detection of blaDHA gene in 49.02% of *E. coli* DNA samples, these antimicrobials cannot be recommended to treat infections caused by *E. coli* [21].

The conducted research reveals high resistance to carbapenems (10.22%), which is a bad prognosis confirming general epidemiological trend towards spread of microorganisms resistant to these antimicrobials. It should be emphasized that carbapenems are used in medicine only as reserve antimicrobials [22]. The absence of VIM gene does not exclude other carbapenemase-producing genes.

In general, the tendency to develop resistance to carbapenems and third-generation cephalosporins is a marker of polyresistance of bacteria belonging to the *Enterobacteriaceae* family [23].

The recovered isolates turned out to be sensitive to antimicrobials included into combined pharmaceuticals. However, their widespread use poses a risk of developing cross-resistance to antimicrobials from different groups and polyresistance.

The results of studying phenotypic and genotypic characteristics of antimicrobial resistance in *E. coli* clinical isolates recovered from cattle in the Ural region are important for a systematic approach to rational drug use and to AMR control and containment in livestock production.

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Study of resistance of pathogenic and opportunistic fungi to antimycotics

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SUMMARY

The widespread use of antimycotic agents for the treatment of mycosis in humans and animals highly concerns medical and veterinary specialists, due to the emergence of resistance in pathogenic and opportunistic fungi to antifungal agents. In recent years, information on the various molecular mechanisms underlying this phenomenon has been published, but in-depth studies are still needed to successfully predict the occurrence of resistance in various groups of fungi. To treat and prevent fungal infections, several groups of antimycotics are used, with azoles and allylamines being the most frequent ones. This, however, leads to the development of resistance in pathogenic and opportunistic fungi. The article presents the results of molecular identification methods of azole-resistant *Candida albicans* isolates and terbinafine-resistant *Trichophyton* isolates. The analysis of *ERG11* gene nucleotide sequences of 10 *Candida albicans* isolates, recovered from different animal species, enabled the division of phenotypically resistant and susceptible strains, but could not differentiate between strains that have dose-dependent resistance to azoles. The study of single nucleotide polymorphisms in the SQLE gene, that are associated with the development of resistance to terbinafine in 12 fungal isolates of the genus *Trichophyton*, did not allow to classify them by their resistance, which is likely associated with another resistance mechanism that can be observed in these strains. The obtained results can serve as a basis for using molecular methods for characterization of fungi belonging to *Candida* and *Trichophyton* genera. However, taking into the account biological features of pathogens from different groups, it would be reasonable to use several significant genome regions or the results of the whole genome sequencing, as well as the gene expression analysis, to predict the development of resistance.

Keywords: antimycotic resistance, *Candida*, *Trichophyton*, azoles, terbinafine, polymerase chain reaction, sequencing

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Изучение резистентности патогенных и условно-патогенных грибов к противогрибковым препаратам

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РЕЗЮМЕ

Широкое применение антимикотических средств для терапии микозов у человека и животных вызывает беспокойство медицинских и ветеринарных специалистов в связи с возникновением резистентности патогенных и условно-патогенных грибов к противогрибковым препаратам. За последние годы накоплена информация о различных молекулярных механизмах, лежащих в основе данного явления, однако для успешного прогнозирования резистентности

в различных группах грибов необходимо провести углубленные исследования. Для терапии и профилактики грибковых заболеваний активно применяют несколько групп препаратов, среди которых наиболее часто используют азолы и аллиламины, что приводит к накоплению резистентности патогенных и условно-патогенных грибов к этим противогрибковым средствам. В работе представлены результаты использования молекулярно-генетических методов для выявления устойчивых к азолам изолятов *Candida albicans* и устойчивых к тербинафину изолятов грибов рода *Trichophyton.* Анализ нуклеотидных последовательностей гена *ERG11* 10 изолятов *Candida albicans*, выделенных от разных видов животных, позволил разделить фенотипически устойчивые и чувствительные штаммы, однако не дал возможности дифференцировать штаммы, обладающие дозозависимой устойчивостью к азолам. Изучение однонуклеотидных полиморфизмов в гене SQLE, ассоциированном с развитием устойчивости к тербинафину, у 12 изолятов грибов рода *Trichophyton* не позволило разделить их по резистентности, что, вероятно, связано с действием другого механизма устойчивости, который может наблюдаться у данных штаммов. Полученные результаты исследований служат основанием для использования молекулярно-генетических методов для характеристики грибов рода *Candida* и *Trichophyton*, однако, с учетом биологических особенностей патогенов разных групп, для прогнозирования резистентности целесообразно использовать несколько значимых участков генома или результаты полногеномного секвенирования, а также анализ экспрессии генов.

Ключевые слова: антимикотическая устойчивость, *Candida*, *Trichophyton*, азолы, тербинафин, полимеразная цепная реакция, секвенирование

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INTRODUCTION

In recent years, there has been an unprecedented increase in the development of resistance to antimycotic agents in parasitic fungi, which cause severe diseases in humans. The risk group primarily includes people with weakened immune system. International organizations have called for increasing the intensity of research and control of the resistance to antifungals, also including the field of veterinary medicine. The spread of resistance in the population is a serious problem, since some socially significant fungal infections are transmitted from animals to humans, and vice versa.

The use of antifungals to treat mycosis in animals in the Russian Federation is spontaneous and is not regulated. This fact, certainly, contributes to the development of resistance to antimycotics in pathogenic and opportunistic fungi.

It is assumed that the widespread use of antifungal agents contributes to the development of drug resistance [1, 2]. With this being said, fungal resistance to antimycotic drugs is becoming a serious problem on a global scale.

Currently existing variants of antifungals are represented by several classes of substances that differ in both, chemical composition and the mode of action (Table 1).

The drugs usually affect plasma membrane, cell wall, nucleic acids, and fungal mitosis. Nowadays for public and animal health purposes, azoles (for example, fluconazole, voriconazole and posaconazole) and allylamines (for example, terbinafine) are mostly used for treatment and prevention of mycosis, caused by the fungi of genera *Candida*, *Microsporum,* and *Trichophyton*. As a result, in most cases resistance develops to these specific drugs [1, 3].

The resistance of *Candida* fungi to azoles is developed mainly due to the following mechanisms: changes in the drug targets, decrease in the intercellular concentration of the target enzyme, changes in the sterol biosynthetic pathway, overexpression of the antifungal target, or increased efflux of the drug across the cell surface. A specific target of azoles is the cytochromes P450-dependent enzyme lanosterol 1,4-*α*-demethylase encoded by the *ERG11* gene in yeast-like fungi. The protein product of this gene catalyzes the removal of the 1,4 *α*-methyl group from lanosterol. Binding of azole to a fragment of ferric iron at the heme-binding site blocks the enzyme's natural substrate, lanosterol, disrupting the biosynthetic pathway [4]. Amino acid substitutions in the drug target are a common mechanism of resistance to azoles in *Candida.* More than 140 substitutions have been reported in the resistant strains, many of which have an additive effect [5]. The most frequent alterations in *C. albicans* occur in two positions: R467K and G464S, which are in the close proximity to the heme-binding site [5, 6].

According to published data, overexpression of the *ERG11* is common among azole-resistant clinical isolates of *C. albicans* [7–9]. This directly contributes to the development of resistance, since an increase in the number of targets requires higher concentrations of the drug for inhibition [8], reducing its overall sensitivity. Overexpression of the *ERG11* has also been reported for azole-resistant isolates of other *Candida* species – *C. glabrata*, *C. parapsilosis*, *C. tropicalis* and *C. krusei* [10–14]. The mechanism of overexpression in these species and its contribution to azole resistance remains largely unknown.

The most common mechanism of fungal resistance is the activation of membrane efflux pumps, which recognize

Table 1
Classification of major antifungals (according to A. K. Sahoo et al.) [3]

Group	Examples	Mechanism of action
Polyenes	nystatin, levorine, natamycin, amphotericin B	Fungistatic and fungicidal action by binding of the drug to ergosterol on the fungal cell membrane and thus destabilizing it
Azoles: *Imidazoles* *Triazoles*	ketoconazole, clotrimazole, miconazole, bifonazole, econazole, isoconazole, oxiconazole itraconazole, fluconazole, voriconazole, posaconazole, ravuconazole, pramiconazole, albaconazole	Fungistatic (less often – fungicidal) action by inhibiting 1,4*α*-demethylase that catalyzes the conversion of lanosterol into ergosterol
Allylamines	terbinafine, butenafine, naftifine	Fungicidal action by inhibition of the ergosterol synthesis. Unlike azoles, allylamines block earlier stages of biosynthesis by inhibiting the enzyme squalene epoxidase
Echinocandins	caspofungin, anidulafungin, micafungin, aminocandin	Fungistatic and fungicidal action by inhibiting synthesis of 1,3-*β*-D-glucan in the cell wall
Pyrimidines	flucytosine	Fungistatic and fungicidal effects associated with inhibited nucleic acid/protein synthesis
Sordarin derivatives	GR135402, GM237354	
Drugs of different groups		
	griseofulvin	Fungistatic effect by inhibition of cellular mitotic activity in metaphase and disruption of DNA synthesis
	amorolfine	Fungistatic and fungicidal action caused by damage of the fungal cell membrane structure
	nikkomycin	Fungistatic and fungicidal action by blocking chitin formation

various chemicals and contribute to multidrug resistance. Fungi have several different drug efflux systems that are encoded by at least ten different genes. Mutations in each of these genes can also affect the degree of pathogen resistance to the drug. In addition, multiple genomic alterations are associated with azole resistance, such as loss of heterozygosity in certain genomic regions, increase in the number of copies of chromosomes, as well as segmental or chromosomal aneuploidies [15]. The loss of heterozygosity is common for the regions containing genes that determine azole resistance, including *ERG11*. Analysis of *C. albicans* isolates with developed resistance revealed that mutations in these genes often arise in the heterozygous state and, then, become homozygous [16]. The prevalence of aneuploidies in azole-resistant isolates raised the question of whether azole exposure simply selects for the more resistant aneuploid variants or whether azole exposure contributes to the development of aneuploidies. It was found that the exposure to azole initiates aberrant cell cycle regulation with a tetraploid intermediate, which, ultimately, precedes the formation of aneuploidy in *C. albicans* [17].

It must be said that nowadays, the gene expression analysis is most widely used to study azole-resistance mechanisms. The technique is based on the comparison of the expression levels of certain genes in azole-susceptible and azole-resistant strains. However, to carry out this analysis, it is necessary to have fresh *C. albicans* cultures grown on a nutrient medium under the same conditions.

Thus, the development of resistance to azoles is a complex process that involves different biochemical processes and gene combinations.

Allylamines is another group of drugs, to which resistance is often evolved. Among its representatives, terbinafine is most actively used worldwide to treat many diseases caused by different species of dermatophyte fungi. The terbinafine's target is squalene epoxidase (SQLE), responsible for ergosterol biosynthesis that catalyzes the conversion of squalene into 2,3-oxidosqualene. Terbinafine inhibits squalene oxidase activity, resulting in ergosterol depletion and squalene accumulation [18]. Mutations in the SQLE gene that give rise to amino acid substitutions lead to structural changes and decreased binding ability of terbinafine to the protein without causing dysfunction in ergosterol biosynthesis [19].

The mechanism of resistance to this allylamine among *Trichophyton* species is attributed to single nucleotide polymorphism (SNP) mutations in the SQLE gene. Point mutations in the SQLE gene lead to single amino acid substitutions at one out of four positions (Leu^{393}, Phe^{397}, Phe^{415}, His^{440}), which corresponds to the increased values of minimum inhibitory concentrations (MICs) for terbinafine *in vitro* [20]. On the contrary, newly occurring missense mutations in the SQLE gene lead to H440Y/F484Y and I121M/V237I substitutions, which were detected in low-resistant isolates [21].

In the study by Yamada T. et al., the group tested 2,056 isolates of *T. rubrum* and *T. interdigitale* for their susceptibility to terbinafine. The authors found that only 17 of them (less than 1%) were resistant to this antifungal agent. By analyzing the genetic sequence of the SQLE gene, they found point mutations in four positions responsible for the exhibiting resistance phenotype. In addition

to L393F and F397L, seven new mutations were identified, including one in the L393 residue and two in the F397 residue. These mutations were studied through the expression of the corresponding amino acid substitutions using *T. mentagrophytes* as a recipient organism. The strains that harbored mutated genes were less susceptible to terbinafine. At the same time, proteomics revealed no other significant differences between the mutated strains and the ones in the control group, indicating that the increased terbinafine resistance was caused by mutations [20].

The aim of the work was to evaluate the possibility of identifying azole-resistant strains of *C. albicans* and terbinafine-resistant strains of *Trichophyton* spp. by using molecular-genetic methods without assessing the expression levels of certain genes.

MATERIALS AND METHODS

In the study, ten isolates of *C. albicans* were taken from cattle and dogs, isolates of *T. interdigitale* and *T. rubrum* were taken from nail plates of individuals with onychomycosis signs, isolates of *T. verrucosum* were taken from the cattle wool, and isolates of *T. mentagrophytes* were taken from the coat of dogs with the signs of dermatomycosis.

The susceptibility (MICs) of *C. albicans* to fluconazole (FLU), itraconazole (ITR), and voriconazole (VRC) was determined in accordance with EUCAST E.Def 7.3.2 [22]. The MICs were interpreted in accordance with Breakpoint tables for interpretation of MICs for antifungal agents, version 10.0, 2020 (FLU: MIC more than 4.0 mg/L – resistant, from 2.0 to 4.0 mg/L – susceptible at increased exposure, less than 2.0 mg/L – susceptible; ITR: MIC more than 0.06 mg/L – resistant, less than 0.06 mg/L – susceptible; VRC: MIC more than 0.25 mg/L – resistant, from 0.06 to 0.25 mg/L – susceptible at increased exposures, less than 0.06 mg/L – susceptible) [23].

The isolate was identified as "resistant" or "sensitive" to azoles using the following model: an isolate resistant to three drugs was considered as resistant; an isolate resistant or showing intermediate resistance to one or two drugs was considered was considered to exhibit dose-dependent sensitivity; an isolate susceptible to all drugs was considered as sensitive to azoles.

Data on *C. albicans* isolates, their susceptibility to azole drugs, and characterization of their susceptibility are given in Table 2.

The susceptibility of *Trichophyton* to terbinafine (TBF) was determined by the method developed in the Department of Mycology in FSBI "VGNKI", based on EUCAST E.Def 9.3.1 [24]. Susceptibility was interpreted on the basis of MIC ranges for terbinafine (mg/L): more than 32 mg/L – resistant, from 16 to 32 mg/L – exhibit dose-dependent sensitivity, less than 16 mg/L – sensitive.

Data on *T. verrucosum*, *T. mentagrophytes*, *T. interdigitale* and *T. rubrum* isolates, their susceptibility to antifungal drugs and characterization of their susceptibility to the drug are given in Table 3.

DNA was extracted using a commercial kit "DNA-sorb-C-M", and amplification was performed using reagents manufactured by AmpliSens® (FBIS CRIE, Russia) by "Tertsik" machine (DNA Technology LLC, Russia).

To amplify DNA of *C. albicans,* primers described earlier by M.-K. Lee et al. [25] were used. To identify *Trichophyton* resistance to terbinafine, the following selected primers for amplification of the informative site of the SQLE gene were used: Tr-terb-F 5′-CTTAGTCCAGAGGCCGTACC-3′ and Tr-terb-R 5′-AGGATGACCCTGCAGGCAGT-3′. Cycles of polymerase chain reaction for *Trichophyton* isolates were as following: one cycle at 95 °C for 5 min, then 42 cycles at 95 °C for 10 sec., 60 °C for 10 sec., 72 °C for 10 sec., then one cycle at 72 °C for 1 min.

The Sanger sequencing was performed for the amplification of fragments using ABI PRISM Big Dye v. 1.1 cycle

Table 2
Data on *Candida albicans* isolates used in the study

Isolate	Source	Minimum inhibitory concentration, mg/L			Characterization
		FLU	ITR	VRC	
C. albicans No. 1	cattle, udder	4.0	0.125	0.5	resistant
C. albicans No. 2	cattle, udder	4.0	0.125	0.5	resistant
C. albicans No. 3	cattle, udder	8.0	0.25	0.5	resistant
C. albicans No. 4	cattle, milk	8.0	0.125	0.5	resistant
C. albicans No. 5	dog, stomatitis	4.0	0.125	0.5	resistant
C. albicans No. 6	dog, stomatitis	1.0	0.03	0.03	sensitive
C. albicans No. 7	cattle, milk	2.0	0.06	0.25	dose-dependent
C. albicans No. 8	cattle, udder	2.0	0.06	0.25	dose-dependent
C. albicans No. 9	cattle, udder	1.0	0.03	0.03	sensitive
C. albicans No. 10	cattle, milk	2.0	0.06	0.125	dose-dependent

Table 3
Data on dermathophyte isolates used in the study

No.	Isolate	Source	Minimum inhibitory concentration TBF, mg/L	Characterization
1	*T. verrucosum* No. 1	cattle wool	16	dose-dependent
2	*T. verrucosum* No. 2	cattle wool	16	dose-dependent
3	*T. verrucosum* No. 3	cattle wool·	16	dose-dependent
4	*T. verrucosum* No. 4	cattle wool·	16	dose-dependent
5	*T. mentagrophytes* No. 1	dog's coat	64	resistant
6	*T. mentagrophytes* No. 2	dog's coat	32	dose-dependent
7	*T. mentagrophytes* No. 3	dog's coat	32	dose-dependent
8	*T. interdigitale* No. 1	human nails	64	resistant
9	*T. interdigitale* No. 2	human nails	64	resistant
10	*T. interdigitale* No. 3	human nails	64	resistant
11	*T. interdigitale* No. 4	human nails	8	sensitive
12	*T. interdigitale* No. 5	human nails	16	dose-dependent
13	*T. rubrum* No. 1	human nails	64	resistant
14	*T. rubrum* No. 2	human nails	64	resistant
15	*T. rubrum* No. 3	human nails	64	resistant

sequencing kit (Applied Biosystems, USA), according to the manufacturer's instructions with the use of automated capillary sequencer ABI-3100 PRISM Genetic Analyzer (Applied Biosystems, USA). The obtained chromatograms were analyzed using the Chromas software. The nucleotide sequences were analyzed using the Vector NTI Advance 11.5.0 program.

The obtained nucleotide sequences of *C. albicans* were compared with X13296 sequence from the GenBank database, which was recognized as a reference sequence for fluconazole-susceptible isolate in the article by M.-K. Lee et al. [25]

The obtained nucleotide sequences of the *Trichophyton* genome fragment were checked for the presence of point mutations responsible for amino acid substitution Leu393Phe, Leu393Ser (nucleotide position 1177 in the gene SQLE), Phe397Leu, Phe397Ile, Phe397Val (nucleotide position 1189 in the gene SQLE), Phe415Ile, Phe415Ser, Phe415Val (nucleotide position 1305 in the gene SQLE), His440Tyr (nucleotide position 1380 in the gene SQLE) [20].

RESULTS AND DISCUSSION

Analysis of C. albicans resistance to azoles. Nucleotide sequences of the *ERG11* gene were obtained for all 10 isolates of *C. albicans*. The resistance of *C. albicans* to azole drugs was evaluated, based on the analysis of both nucleotide and amino acid sequences of the *ERG11* gene.

The obtained results of comparing the isolate nucleotide sequences with the reference sequence are presented in Table 4.

Substitutions in only 10 out of 30 analyzed nucleotide positions in the *ERG11* gene lead to changes in the amino

Table 4
Amino acid and nucleotide substitutions in the *ERG11* gene of *C. albicans* strains

Amino acid substitution	Reference nucleotide in X13296	Isolates of *C. albicans*									
		1	2	3	4	5	6	7	8	9	10
		R	R	R	R	R	S	M	M	S	M
F105F	T	C	C	C	C	C	.	C	C	.	C
D116E	T	.	**A**	**A**	**A**	**A**	.	**A**	.	.	.
K119K	A	.	G	G	G	G	.	G	.	.	.
K128T	A	.	.	C	.	C	.	C	.	.	.
S137S	C	.	.	.	.	.	.	.	.	.	.
K143R	A	.	.	.	.	.	.	.	.	.	.
H183H	T	.	.	.	.	.	C	C	C	C	C
L220L	C	T	T	T	T	T	T	T	T	T	T
E266D	A	**C**	**C**	.	**C**	.	.	.	**C**	.	**C**
L320L	G	.	.	.	.	.	.	.	.	.	.
V332V	T	N	.	.	.	.	C	.	C	C	C
L340L	A	.	G	.	.	G	.	G	.	.	.
K342K	A	G	.	.	.	.	.	.	G	.	G
S361S	A	.	.	.	.	.	G	.	.	G	.
L370L	C	T	T	T	T	T	T	T	T	T	T
F380F	T	.	.	.	.	.	.	.	.	.	.
Y401Y	T	.	.	.	.	.	.	.	.	.	.
V404I	G	.	.	.	.	.	.	.	.	.	.
P419P	T	.	.	.	.	.	.	.	.	.	.
D428D	T	.	.	.	.	.	.	.	.	.	.
V437I	G	.	.	.	.	.	.	.	.	.	.
G448G	G	.	.	.	.	.	.	.	.	.	.
F449V	T	.	.	.	.	.	.	.	.	.	.
V452V	T	.	.	.	.	.	.	.	.	.	.
V456V	T	.	.	.	.	.	.	.	.	.	.
R467K	**G**	.	.	.	.	.	.	.	.	.	.
Q474K	**C**	.	.	**A**	.	**A**	.	.	**A/C**	.	**A/C**
L480L	A	.	G	G	G	G	G/A	G	G/A	G	G/A
N490N	T	.	C	C	C	C	C/T	C	C/T	C	C/T
V509M	G	.	.	.	.	.	.	.	.	.	.

Positions that lead to amino acid substitutions are emphasized in bold.
R – azole-resistant; S – azole- sensitive; M – exhibit dose-dependent sensitivity

acid sequence. Among studied *C. albicans* isolates, no amino acid substitutions were observed in azole-susceptible isolates № 6 and № 9 in this gene region, and in four out of five isolates, substitutions in the amino acid sequence were observed in 2 out of 10 positions. The most informative positions were **D116E** and **E266D**.

As indicated in the Table 4, the obtained data generally corresponds to declared phenotypic resistance of the strains. Interestingly, even a single amino acid substitution in the *ERG11* gene, detected in isolate № 1, was associated with the development of resistance to azole drugs. However, the study failed to differentiate fully azole-resistant and dose-dependent strains. In dose-dependent isolates two substitutions were also identified: one in position **E266D** and the other in position **Q474K** in isolates № 8 and № 10 in the heterozygous state.

Marichal P. et al. in their work [26] identified three "hotspot regions" in the amino acid sequence of the *ERG11* gene, based on the compilation of the *ERG11* mutations, which are reported to be associated with azole-resistance. These "hot spots" include amino acid regions 105–165, 266–287, and 405–488. The detected nucleotide substitutions fall within these "hotspot regions", which can serve as the evidence of the reliability of the obtained results.

Analysis of resistance to terbinafine in *Trichophyton* spp. For this part, primers were selected for the amplification and subsequent sequencing of the 440 bp SQLE gene fragment, covering all positions of single-nucleotide substitutions leading to the development of resistance to terbinafine. 12 *Trichophyton* isolates, both resistant and sensitive to terbinafine, were analyzed, based on mycological test results.

The analysis of obtained chromatograms and multiple sequence alignment using the Vector NTI Advance 11.5.0 program, all isolates were identified as terbinafine-sensitive. In 2 out of 12 isolates, a point mutation was detected at the position 1,177 (substitution of TTA with CTA), but it is not significant and does not lead to amino acid substitution.

The discrepancy in the data on terbinafine resistance obtained by molecular-genetic and cultural methods can be explained by the appearance of additional resistance mechanisms, which can be observed in the strains. This hypothesis is confirmed by the numerous studies of the fungal resistance to antimycotic agents. For example, azole resistance in clinical isolates of *C. albicans* is associated with several mechanisms, including missense mutations in the *ERG11* and overexpression of genes encoding carrier proteins of several drugs. Combined effects of such mechanisms were observed in the same clinical isolate resistant to azoles [27]. It is possible that overexpression of genes encoding efflux pumps is also involved in terbinafine resistance in the studied isolates.

CONCLUSION

The widespread use of azole and allylamine compounds in the treatment and prevention of mycoses contributes to the emergence of strains and isolates resistant to these antifungal drugs. The resistance is conventionally tested using cultural methods, however, for certain types of fungi that are important in the field of veterinary medicine, there are no standards and control points used for classification of isolates into "susceptible" or "resistant", which makes it difficult to interpret the clinical effects of a minimum inhibitory concentration. The current goal is to find reliable methods for detecting resistance, based on the genetic analysis. This paper describes methods for detecting the resistance of *C. albicans* to azoles and of *Trichophyton* dermatophytes to terbinafine by analyzing certain genes. It should be noted that the results obtained so far indicate that the analysis of nucleotide sequences of *C. albicans ERG11* gene and SQLE gene of *Trichophyton* fungi has limited informative value for predicting resistance to antifungal drugs (azoles and terbinafine, respectively). The obtained information on the structure of these genes can be used for the characterization of strains. However, in order to increase the reliability of molecular-genetic tests for resistance, it is necessary in the future studies to use more fungal isolates characterized phenotypically, and include several genes associated with antimycotic resistance in the analysis.

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Antimicrobial resistance of *Salmonella* isolates recovered from animal products

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SUMMARY

The article provides data on antimicrobial resistance (AMR) of *Salmonella* isolates recovered from animal products tested in the Laboratory for Microbiological Testing of the FGBI "ARRIAH" from 2019 to 2020. 106 isolates of *Salmonella enterica* subsp. *enterica* were recovered from 4,500 tested samples of raw materials and products of animal origin, 23% of them were untyped, and 77% belonged to 17 serological variants. Isolates of *S. enteritidis* ($n = 37$) and *S. virchow* ($n = 9$) serovariants dominated among the typed cultures of *Salmonella*, which is consistent with the data from other authors. Antimicrobial susceptibility of the microorganisms was determined in a disk diffusion test in accordance with the recommendations of the European Committee on Antimicrobial Susceptibility Testing. Different *Salmonella* serovars demonstrated different proportions of susceptible and resistant isolates, in terms of antibiotics from ten pharmacological groups. The largest number of polyresistant isolates was noted in *Salmonella* serovars *S. virchow*, *S. nigeria*, *S. infantis*, *S. colindale*. Both resistant and polyresistant *Salmonella* isolates were most often isolated from poultry products. *S. typhimurium* serovar, which is referred to in literature as polyresistant, was resistant to one or two antimicrobial agents as the research demonstrates. Isolates of 9 *Salmonella* serovars out of 17 (65%) showed resistance to nalidixic acid. 97% ($n = 36$) of *S. enteritidis* isolates were resistant to this antimicrobial agent. Isolates of *S. colindale* serovar ($n = 2$) were resistant to 8 antimicrobials, *S. papuana* ($n = 5$) – to 6 antibiotics, and *S. agona* ($n = 3$) – to 5 antimicrobials. Untyped *Salmonella* isolates were resistant to 9 antibiotics, 2 cultures out of them showed resistance to ciprofloxacin.

Keywords: *Salmonella*, serovariant, antibiotic, susceptibility, resistance

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Антибиотикорезистентность изолятов сальмонелл, выделенных из продуктов животного происхождения

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РЕЗЮМЕ

Представлены результаты изучения антибиотикорезистентности изолятов сальмонелл, полученных при исследовании образцов продуктов животноводства в лаборатории микробиологических исследований ФГБУ «ВНИИЗЖ» за период с 2019 по 2020 г. При испытании 4500 проб сырья и продукции животного происхождения было выделено 106 изолятов бактерий *Salmonella enterica* subsp. *enterica*, из которых 23% были нетипируемыми, а 77% принадлежали к 17 серологическим вариантам. Среди типированных культур сальмонелл доминировали изоляты сероваринтов *S. enteritidis* ($n = 37$) и *S. virchow* ($n = 9$), что согласуется с данными других авторов. Чувствительность микроорганизмов к антибактериальным препаратам определяли диско-диффузионным методом, в соответствии с рекомендациями European Committee on Antimicrobial Susceptibility Testing. Были выявлены различия в соотношении чувствительных и резистентных изолятов бактерий рода *Salmonella* разных серологических вариантов по отношению к антибиотикам десяти фармакологических групп. Наибольшее число полирезистентных изолятов отмечали у сальмонелл сероваров *S. virchow*, *S. nigeria*, *S. infantis,*

S. colindale. Резистентные и полирезистентные изоляты сальмонелл наиболее часто выделяли из продукции птицеводства. Сероваp *S. typhimurium*, который в источниках литературы определяют как полирезистентный, в представленных исследованиях был устойчив к одному или двум антимикробным препаратам. У изолятов 9 сероваров сальмонелл из 17 (65%) отмечена устойчивость к налидиксовой кислоте. Доля резистентных к данному средству изолятов *S. enteritidis* составила 97% ($n = 36$). Изоляты серовара *S. colindale* ($n = 2$) были устойчивы к 8 антимикробным препаратам, *S. papuana* ($n = 5$) – к 6 антибиотикам, а *S. agona* ($n = 3$) – к 5 антибактериальным средствам. Нетипируемые изоляты сальмонелл были резистентны к 9 антибиотикам, из которых 2 культуры проявили устойчивость к ципрофлоксацину.

Ключевые слова: *Salmonella*, сероваpиант, антибиотик, чувствительность, резистентность

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INTRODUCTION

Salmonellosis is a widespread infection of humans and animals caused by various representatives of *Salmonella enterica* [1–3]. According to the government report "On sanitary and epidemiological well-being of the population in the Russian Federation in 2020", the overall incidence of salmonellosis showed a decrease. Compared with 2019, the 2020 incidence decreased by 1.6 times and accounted for 14.71 cases per 100 thousand [4, 5]. However, salmonellosis is still a matter of concern. Based on the data provided by the reference center for monitoring of salmonellosis, 27 *Salmonella* serotypes were isolated from infected humans in 2020, 17 serotypes were isolated from food staples, 16 from environmental objects. As in previous years, *Salmonella* was most often isolated from poultry products [4].

Salmonella is susceptible to a wide range of antimicrobials, due to the structural features of the cell wall. As Gram-negative microorganisms, the bacteria are susceptible to: beta-lactams (aminopenicillins, carboxypenicillins, inhibitor-protected penicillins, cephalosporins, monobactams, carbapenems), aminoglycosides (streptomycin, kanamycin, gentamicin, tobramycin, amikacin), quinolones (nalidixic acid) and fluoroquinolones (ciprofloxacin, norfloxacin, ofloxacin, etc.), tetracyclines (tetracycline, doxycycline), polymyxins, sulfonamides and co-trimoxazole, nitrofurans (nifuroxazide, nifuratel, furazolidone) and products of other groups (fosfomycin, chloramphenicol) [6–8].

According to the report "On sanitary and epidemiological well-being of the population in the Russian Federation in 2020", from 2015 to 2020, the following serotypes were predominantly recovered: *S. enteritidis* (64.7%), *S. typhimurium* (4.8%) and *S. infantis* (3.2%). A study of antimicrobial susceptibility of *S. enteritidis* isolates recovered in 2020 revealed that 58.7% of cultures are resistant to colistin, and 75% – to ciprofloxacin. Polyresistance was noted in 4.1% of isolates, and two cultures of *S. enteritidis* demonstrated resistance to polymyxins, monobactams, penicillins, second-, third-, forth-generation cephalosporins. 85% of the isolated *S. infantis* cultures showed resistance to more than three classes of antibiotics, while all the isolates were susceptible to glycocyclines, polymyxins, carbopenems, first-generation cephalosporins and third-generation aminoglycosides. About half of *S. typhimurium* isolates were susceptible to all antimicrobials, and 30% of cultures were resistant to two groups of antibiotics (penicillins, tetracyclines) [4].

The World Health Organization estimates that half of all the antibiotics produced in the world are used to not only treat people, but also those animals and birds who are used to produce products for human consumption. Consequently, the number of strains resistant to antimicrobials is steadily increasing [9].

The issue of antimicrobial resistance has now become extremely alarming in Russia, as well as in the EU, the USA, Canada, etc., and in order to solve it, the Government of the Russian Federation developed the "Strategy for preventing spread of antimicrobial resistance in the Russian Federation until 2030", approved by Order No. 2045-r of the Government of the Russian Federation, September 25, 2017 [10].

According to the WHO, *S. enterica* bacteria belongs to the microorganisms that have resistant serotypes found in the food chain [11].

Such antimicrobials as chloramphenicol, ampicillin, and co-trimoxazole were used at different historical periods of human and veterinary medicine to treat salmonellosis. In the years that followed, the pathogen developed resistance to traditional medicinal products, therefore, fluoroquinolones and extended-spectrum cephalosporins (ESC) were adopted for treatment of salmonellosis [12]. Currently, the *Enterobacteriaceae* family demonstrates a steadily increasing resistance to most antimicrobials used for treatment of infectious diseases in animals and humans. In this regard, it is mandatory to determine antimicrobial susceptibility of bacteria isolated not only from affected animals, but also from animal products. European countries and the USA ensure continuous control over antimicrobial resistance in microorganisms, including *Salmonella*. First,

they monitor resistance to quinolones and ESP, and polyresistance to antimicrobials [13, 14]. Therefore, detection of *Salmonella* isolates resistant to antimicrobials in animal products is an urgent problem.

The purpose of this work was to determine antimicrobial resistance of *Salmonella* isolates recovered from raw materials of animal origin from 2019 to 2020.

MATERIALS AND METHODS

Strains of microorganisms. Strains *Salmonella typhimurium* ATCC 14028 and *Escherichia coli* ATCC 25922 deposited in the collection were used to control nutrient media and *Salmonella* isolation methods.

Isolates of bacteria. 106 *Salmonella* isolates recovered from animal products and food products for microbiological tests in 2019–2020 were used in the work.

Antibiotics. To determine the antimicrobial susceptibility of bacteria, standard paper disks with the following antibiotics were used: azithromycin (15 μg), nalidixic acid (30 μg), streptomycin (10 μg), tetracycline (30 μg), amikacin (30 μg), amoxicillin (20 μg), trimethoprim/sulfamethoxazole (23.75/1.25 μg), ampicillin (10 μg), gentamicin (10 μg), cefotaxime (30 μg), levomycetin (30 μg), imipenem (10 μg), meropenem (10 μg), ciprofloxacin (5 μg), kanamycin (30 μg) (Saint Petersburg Pasteur Institute, Russia).

Salmonella isolation and identification. The bacteria were isolated from samples of animal products and food products according to GOST 31659-2012 "Food products. Method for the detection of *Salmonella* spp.",[1] using nutrient media produced by the FBIS SRCAMB (Russia).

For non-selective pre-enrichment, a sample of the product weighing (25 ± 0.1) g prepared for the study was put into a sterile bag containing 225 cm^3 of buffered peptone water and was homogenized for 1 min. The inocula were incubated at a temperature of (37 ± 1) °C for (18 ± 1) hours. After the pre-enrichment, 1 cm^3 samples were inoculated into test tubes with 10 cm^3 of RVS (Rappaport – Vassiliadis Medium) and 10 cm^3 of selenite medium. Inocula on RVS were incubated at a temperature of (41.5 ± 1) °C, and on a selenite medium – at (37 ± 1) °C for (24 ± 1) hours. After incubation, the content of each tube was streaked with a loop on two media: bismuth-sulfite-agar (BSA) and XLD (xylose-lysine-deoxycholate agar). The inocula were incubated at a temperature of (37 ± 1) °C for (24 ± 1) hours. The fact that the grown colonies belonged to *Salmonella* was confirmed by enzyme immunoassay using Singlepath® rapid tests (Merck KGaA, Germany) and mini VIDAS analyzer (bioMerieux SA, France).

Serological identification of Salmonella. The serological formula of the isolated *Salmonella enterica* cultures was determined in agglutination test on glass with diagnostic mono- and polyvalent O- and H-sera adsorbed for the PETSAL® test (FSUE SPbSRIVS FMBA, Russia). The serological variant of the strain was determined based on the serological formula in accordance with the Kaufman – White scheme.

Determination of antimicrobial susceptibility. The identified cultures were tested for antimicrobial susceptibility using disk diffusion test according to MUC 4.2.1890-04 "Determination of the antimicrobial susceptibility of microorganisms"[2] and clinical recommendations "Determination of antimicrobial susceptibility of microorganisms"[3]. The bacterial suspension (0.5 according to the McFarland turbidity standard) was evenly distributed on the surface of the Mueller – Kauffman agar. Disks with antibiotics were put on the surface of agar inoculated with the tested culture (maximum 6 disks per 1 dish). After the disk application, Petri dishes were placed upside down in a thermostat and incubated at a temperature of (35 ± 1) °C for 18–24 hours.

The results were evaluated by microbial growth "inhibition zone" around the disks. The diameter of the microbial growth "inhibition zone", taking into account the diameter of the disk itself, was determined with an accuracy of 1 mm.

Interpretation and analysis of the results. Based on the recommendations of the European Committee on Antimicrobial Susceptibility Testing (EUCAST) the bacterial isolates were divided into the following groups: susceptible, resistant to one and two antibiotics, polyresistant – resistant to three or more antibiotics [15]. The group of resistant isolates included extremely resistant cultures, which were resistant to 6 and 7 antimicrobials [16].

Statistical processing of the test results. The experiments were performed in triplicate. The obtained data were statistically processed in standard Microsoft Exel 2010 analysis package.

RESULTS AND DISCUSSION

From 2019 to 2020, 4,500 samples of animal products were tested in the FGBI "ARRIAH" Laboratory for Microbiological Research in order to detect contamination with *Salmonella*. As a result, 106 isolates were recovered and identified by growth and biochemical properties as *Salmonella* bacteria, which belonged to 17 serological variants from groups B, C, D, E, and 24 isolates of *Salmonella enterica* (which belonged to serogroups B, C and E) were untyped.

The recovered isolates belong to the following serovariants. According to the literature, *S. enteritidis* is the main causative agent of salmonellosis in both animals and humans (35%) [17–19]. Most of the cultures in our work (34.9%) also belonged to this serovariant. Isolates of *S. virchow* serovar were detected in 9 samples (8.5%). Isolates of such serovars as *S. nigeria*, *S. papuana* and *S. infantis* were identified in 7 (6.6%), 5 (4.7%) and 5 (4.7%) samples, respectively (Table). Serotype *S. typhimurium*, clinically important for both animals and humans, was detected in only two samples, which accounted for 1.9%.

Untyped isolates of *Salmonella enterica* subsp. *enterica* were identified in 24 samples of animal products, which accounted for 22.6%. Serovar *S. agona,* which caused two outbreaks of salmonellosis in 2017–2018 in the EU member countries, was isolated in our studies from 3 samples (2.8%) [20].

Determination of Salmonella isolates susceptibility to antibiotics from various pharmacological groups. When choosing antimicrobials to check antimicrobial resistance

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[3] Determination of antimicrobial susceptibility of microorganisms. 2018. 206 p. Available at: https://flm.kz/files/14062184925c1281c1dfd6b.pdf.

Table
Serological variants of *Salmonella* most often recovered from animal products (*n* = 74)

Serological variant	Number of isolates	% of the total number of isolates recovered from food products
S. enteritidis	37	34.9
S. virchow	9	8.5
S. nigeria	7	6.6
S. papuana	5	4.7
S. infantis	5	4.7
S. derby	4	3.8
S. agona	3	2.8
S. colindale	2	1.9
S. typhimurium	2	1.9

of 106 *Salmonella* isolates, we considered the use of these products for human and veterinary medicine and took into account EUCAST recommendations for determination of antimicrobial susceptibility of the Gram-negative microorganisms.

As a result, it was found that *Salmonella* isolates are susceptible to 3 antibiotics from the aminoglycosides group (amikacin – 100%, gentamicin – 100% and kanamycin – 98%), to carbapenems (imipenem – 100% and meropenem – 100%), as well as to cefotaxime – 97%. The susceptibility of *Salmonella* cultures to levomycetin was 92%, azithromycin – 91%, ciprofloxacin – 90%. 87% of isolates were susceptible to ampicillin and 84% to streptomycin (Fig. 1).

Seventy-four percent of isolates demonstrated resistance to nalidixic acid, and 45% of cultures demonstrated resistance to trimethoprim/sulfamethoxazole.

As Figure 2 shows, only ten out of the total number of the tested isolates (*n* = 106) were susceptible to all groups of antimicrobials, which was 9%, whereas 96 isolates (91%) were resistant. At the same time, the proportion of isolates resistant to one group of antimicrobials was 38% (40 isolates).

Twenty cultures (19%) showed resistance to 2 groups of antibiotics. Thirty- six isolates (34%) were polyresistant, i.e. resistant to three or more antimicrobials. This group included extremely resistant isolates, i.e. those that are resistant to 6 antibiotics: ciprofloxacin, streptomycin, nalidixic acid, cefotaxime, levomycetin and kanamycin. Extremely resistant isolates (resistant to 7 antimicrobials) accounted for 4% of the total number of polyresistant.

Determination of antimicrobial resistance in Salmonella isolated from samples of animal products. Most *Salmonella* isolates were recovered from poultry meat (Fig. 3), whereas the proportion of resistant cultures was 90%, including 3 extremely resistant ones. Out of 35 isolates recovered from meat semi-finished products, 32 (91%) were resistant. In the group of isolates recovered from pork and pork semi-finished products, only one out of 13 was susceptible to antimicrobials and one was defined as extremely resistant.

Thus, most polyresistant *Salmonella* cultures were found in poultry meat samples (50%), including extremely resistant ones, alongside with that, most recovered isolates were resistant to many groups of antimicrobials. Isolates resistant to amoxicillin were recovered only from pork samples – 69% (*n* = 9).

It should be noted that polyresistant *Salmonella* isolates were found in all groups of animal products, but in beef samples, their number was the smallest.

When studying antibiotic resistance of 53 *Salmonella* isolates recovered from poultry products, it was found that all the isolates were susceptible to three antibiotics of the aminoglycoside group (gentamicin – 100%, amikacin – 100%, kanamycin – 100%), and the carbapenem antibiotics (meropenem – 100% and imipenem – 100%). In addition, 52 isolates (98%) were found to be susceptible to cefotaxime, 48 isolates (91%) to azithromycin and 49 isolates (92%) to levomycetin (Fig. 4).

Resistance to a quinolone drug (nalidixic acid) was reported in 46 *Salmonella* isolates (87%) recovered from poultry meat samples, and resistance to fluoroquinolones (ciprofloxacin) was reported in 2 isolates.

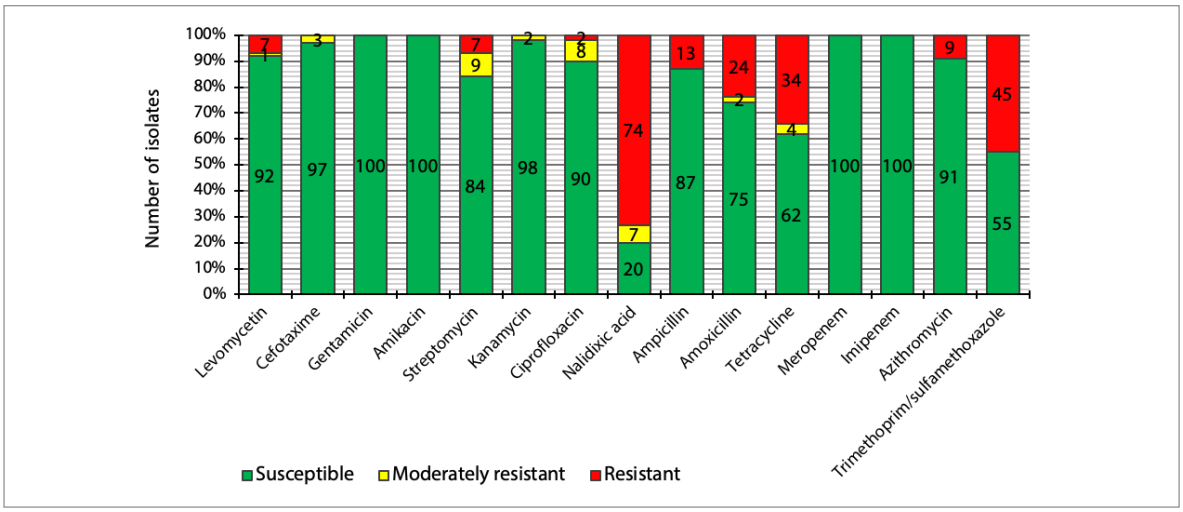

Fig. 1. Antimicrobial susceptibility of Salmonella isolates recovered from raw materials of animal origin

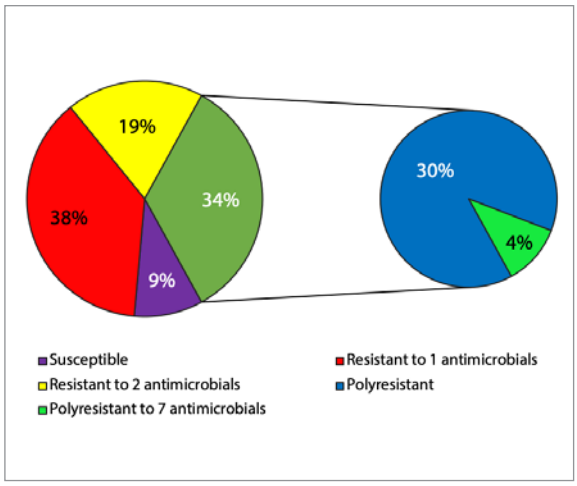

Fig. 2. Proportion of Salmonella isolates susceptible and resistant to antimicrobials (%)

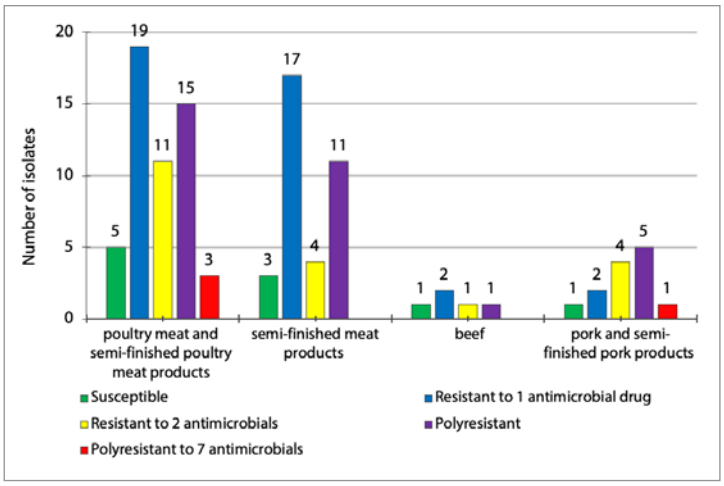

Fig. 3. Antimicrobial susceptibility of Salmonella isolates recovered from products of animal origin

The analysis of antimicrobial resistance in *Salmonella* isolates of various serovariants (Fig. 5) showed that most *S. enteritidis* serovar isolates were resistant to 1 and 2 antibiotics (35 isolates – 95%), only one isolate was polyresistant.

The largest number of polyresistant isolates was observed in the following serovars *S. virchow* (56%), *S. nigeria* (71%), *S. infantis* (40%), and *S. papuana* serovar was represented only by polyresistant isolates (100%), out of which 1 culture was resistant to 5 antimicrobials.

One of *S. colindale* serovar isolates was resistant to 7 antimicrobials.

In the course of the research, it was found that 36 isolates (97%) of *S. enteritidis* serovar are resistant to nalidixic acid, which belongs to the quinolone group and is the highest priority drug included in the WHO list of critically important antimicrobials in human medicine [21]. It is worth noting that resistance to nalidixic acid was also observed in isolates of *S. virchow*, *S. infantis*, *S. nigeria* and *S. papuana*.

Resistance to amoxicillin was reported in 5 isolates of *S. enteritidis* (14%), to trimethoprim/sulfamethoxazole – in 3 isolates (8%) and to tetracycline – in 1 culture (Fig. 5).

All 9 *S. virchow* isolates were resistant to nalidixic acid (100%), and 7 isolates (78%) showed no susceptibility to trimethoprim/sulfamethoxazole. *S. nigeria* isolates ($n = 7$) were 100% resistant to nalidixic acid, and 86% resistant to trimethoprim/sulfamethoxazole. *S. papuana* isolates ($n = 5$) showed resistance to 6 antimicrobials, while all cultures were resistant to trimethoprim/sulfamethoxazole. *S. infantis* isolates ($n = 5$) were resistant to nalidixic acid (4 isolates), to tetracycline (2 isolates) and to trimethoprim/sulfamethoxazole (3 isolates).

Isolates of *S. agona* ($n = 3$) were resistant to amoxicillin, ampicillin, nalidixic acid, streptomycin and tetracycline (Fig. 6). All isolates of *S. colindale* ($n = 2$) were resistant to nalidixic acid (100%) and to 7 antimicrobials. Two isolates of *S. typhimurium* serovar were reported to be resistant to nalidixic acid and azithromycin. Untyped *Salmonella* isolates showed no susceptibility to 9 antibiotics. At the same time, 2 isolates were resistant to ciprofloxacin.

According to the government report "On sanitary and epidemiological well-being of the population in the Russian Federation in 2020", more than 60% of *Salmonella* isolates were found to be resistant to antimicrobials, of which 75% of *S. enteritidis* isolates were resistant to ciprofloxacin [4]. Our study showed that the proportion of isolates resistant to antibiotics was 91%, while 97% of *S. enteritidis* isolates were resistant to nalidixic acid.

It should be noted that a significant part of *Salmonella* isolates are polyresistant. These data are consistent with those ones from the relevant EU reports, which note polyresistance in monophasic variants of *S. typhimurium* (56.7% of isolates), *S. kentucky*, *S. infantis*, *S. typhimurium* and *S. enteritidis*.

Strains isolated from food products are more likely to be polyresistant, unlike strains isolated from humans (41.6% vs. 15.8%). Most of these strains are represented by serovars *S. typhimurium* (over 60%) and *S. infantis*

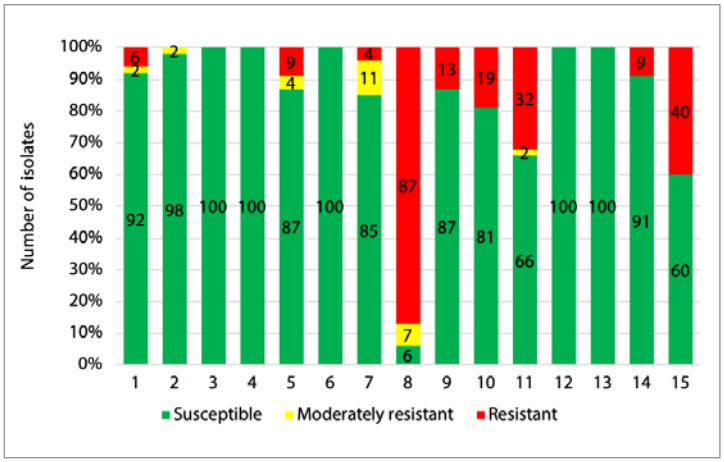

Fig. 4. Antimicrobial resistance of Salmonella isolates recovered from poultry meat and semi-finished poultry meat products:
1 – levomycetin, 2 – cefotaxime, 3 – gentamicin, 4 – amikacin, 5 – streptomycin, 6 – kanamycin, 7 – ciprofloxacin, 8 – nalidixic acid, 9 – ampicillin, 10 – amoxicillin, 11 – tetracycline, 12 – meropenem, 13 – imipenem, 14 – azithromycin, 15 – trimethoprim/sulfamethoxazole

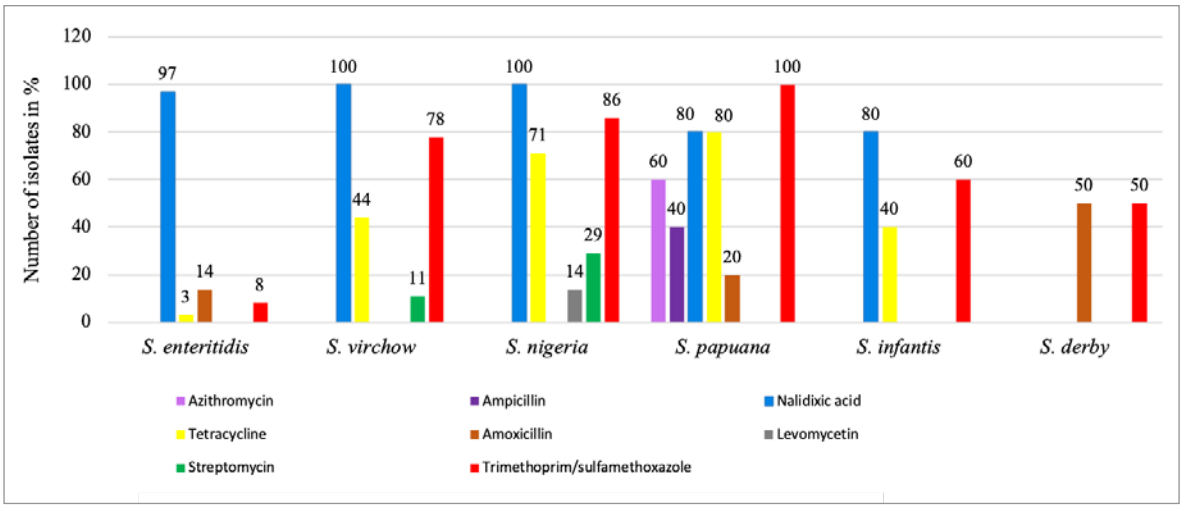

Fig. 5. Resistance of isolates of the following serovars S. enteritidis, S. virchow, S. nigeria, S. papuana, S. infantis and S. derby to antimicrobials

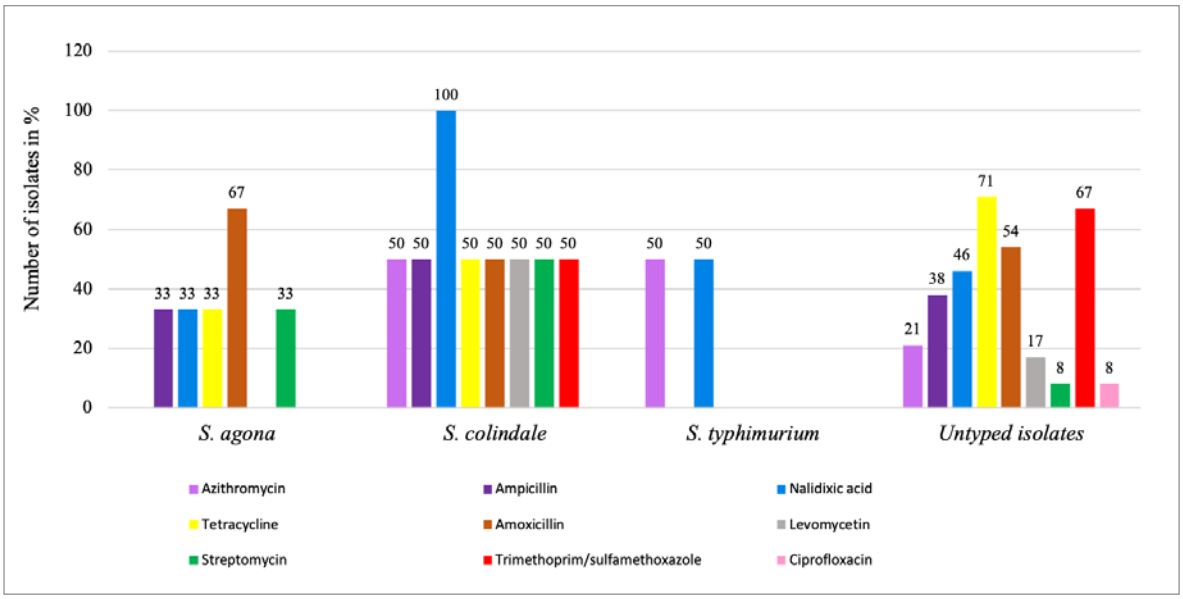

Fig. 6. Resistance of isolates of serovars S. agona, S. colindale, S. typhimurium and untyped cultures to antimicrobials

(over 80%). In general, *Salmonella* resistance to high concentrations of ciprofloxacin has increased [22].

In 2020, isolates of *S. enteritidis* serovariant were most often recovered from animal products in the Russian Federation, which is consistent with the data we obtained. This serovariant was most often recovered (52%) from poultry meat samples, followed by *S. typhimurium* and *S. infantis*, respectively.

Half of *S. typhimurium* isolates was susceptible to all antibiotics, while 30% had resistance to two classes of antimicrobials (penicillins, tetracyclines). Antimicrobial resistance was noted in 90% of *S. infantis* isolates, while resistance to 3 or more medicinal preparations was reported in 59% of them. At the same time, all the studied isolates were susceptible to glycocycline, polymyxins, carbopenems, first-generation cephalosporins and third-generation aminoglycosides [4].

In recent years, there have been reports on *Salmonella* isolates resistant to antimicrobials widely used in human and veterinary medicine, such as sulfonamides (30.5%), tetracyclines (28.8%) and ampicillin (25.9%). The resistance of individual *Salmonella* serovars to these compounds varied from low in *S. enteritidis* (4.5–7.8%) to high in the monophasic variant *S. typhimurium* (86–88%) and *S. kentucky* (71–76%) [23–27].

CONCLUSION

One hundred and six isolates of *S. enterica* subsp. *enterica* were recovered from 4,500 samples of animal products tested in the FGBI "ARRIAH" Laboratory for Microbiological Research from 2019 to 2020. 77% out of the isolates belonged to 17 serological variants, and the rest were untyped (23%). Isolates of *S. enteritidis* serovariants dominated (n = 37) and *S. virchow* (n = 9), which is consistent with the data provided by other authors.

While determining antimicrobial susceptibility of *Salmonella* isolates, we detected differences in the ratio

between susceptible and resistant *Salmonella* of different serological variants in relation to antibiotics of ten pharmacological groups.

Salmonella isolates of all the studied serovariants were found to be susceptible to the following antibiotics: amikacin (100%), gentamicin (100%), kanamycin (98%), imipenem and meropenem (100%).

Salmonella isolates were resistant to quinolones (nalidixic acid) – 74% and to sulfonamides (trimethoprim/sulfamethoxazole) – 45%. The proportion of isolates resistant to one group of antimicrobials was 38%, and 34% were polyresistant, including cultures resistant to 7 antibiotics (4%).

The largest number of polyresistant isolates was noted in *Salmonella* serovars *S. virchow*, *S. nigeria*, *S. infantis*, *S. colindale*, and *S. papuana* serovar was represented only by polyresistant isolates (100%).

Resistant and polyresistant *Salmonella* isolates was most often recovered from poultry products. Cultures resistant to ciprofloxacin were found only in poultry meat. *Salmonella* isolates resistant to nalidixic acid and trimethoprim/sulfamethoxazole were detected in all groups of animal products, and the maximum number of such isolates was recovered from poultry meat – 87% ($n = 46$) and 40% ($n = 21$), respectively. Isolates resistant to amoxicillin were recovered only from pork samples – 69% ($n = 9$).

Serovar *S. typhimurium*, which is known as polyresistant according to the literature, was resistant to one or two antimicrobials in our studies.

Isolates of 9 serovars showed resistance to nalidixic acid, while the proportion of *S. enteritidis* isolates resistant to this product was 97% ($n = 36$). Isolates of *S. colindale* serovar ($n = 2$) were resistant to 8 antimicrobials, isolates of *S. papuana* ($n = 5$) were resistant to 6 antibiotics, isolates of *S. agona* ($n = 3$) – to 5 medicinal products. The untyped *Salmonella* isolates were resistant to 9 antibiotics, including ciprofloxacin (2 isolates).

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Studying formation of *Pseudomonas aeruginosa* biofilms grown under different cultivation conditions

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SUMMARY

The purpose of the present study is to assess how cultivation conditions influence growth and formation of *Pseudomonas aeruginosa* biofilms. The topic is of great importance due to high incidence of *P. aeruginosa*-caused infections and *P. aeruginosa* resistance associated with its ability to form biofilms. The paper analyzes factors that influence biofilm formation, i.e.: growth phase used for inoculation (log, stationary), volume of the growth medium (0.2 and 1.0 ml) and concentration of nutrients (liquid nutrient media diluted to concentrations of 50; 25; 12.5 and 6%) in the cultivation volume. As the research demonstrates, all these factors influence biofilm formation; and a *P. aeruginosa* growth phase before inoculation is a determining factor in the biofilm formation. When *P. aeruginosa* is inoculated at a stationary phase, biofilm formation shows non-linear dependence on concentration of nutrients and on their total amount in the cultivation volume. The linear dependence of biofilm formation on concentration of nutrients in the culture medium is more pronounced, when *P. aeruginosa* is inoculated at a log phase. The study shows that lower concentrations of nutrient media components lead to more noticeable differences in biofilm formation, and such differences are statistically significant. Two-fold dilution of the liquid nutrient medium does not affect the intensity of biofilm formation; however, a 4 to 8-fold decrease in concentration of nutrients in 0.2 ml of cultivation volume inhibited the biofilms formation. In 1.0 ml of the culture medium, the biofilm forms evenly, and in 0.2 ml of 4–8-fold dilution of nutrient medium it grows slower. The slow growth rate is statistically significant. The cultivation volume is also of great importance. For example, cultures grown in 0.2 ml of nutrient medium at different concentrations of nutrients formed fewer biofilms than microorganisms cultivated in 1.0 ml. At the same time, when inoculating *P. aeruginosa* both at log and stationary growth phases, biofilm formation is more pronounced in wells containing more cultivation volume.

Keywords: biofilms, bacteria, *P. aeruginosa*, stationary growth phase, log growth phase

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Изучение формирования биопленок культурой *Pseudomonas aeruginosa* при различных режимах культивирования

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РЕЗЮМЕ

Настоящее исследование посвящено изучению влияния условий культивирования на рост и формирование биопленок культурой *Pseudomonas aeruginosa*. В связи с высокой частотой встречаемости инфекционных заболеваний, вызванных *P. aeruginosa*, а также устойчивостью синегнойной палочки, в особенности из-за способности образовывать биопленки, данная тема не теряет актуальности. В работе проанализировали влияние на феномен биопленкообразования таких характеристик, как используемая для посева фаза роста культуры (логарифмическая, стационарная), объем среды для выращивания (0,2 и 1,0 мл) и концентрация питательных веществ (жидкие питательные среды, разведенные до концентраций 50; 25; 12,5 и 6%) в объеме культивирования. Проведенные исследования показали, что на образование биопленок оказывает влияние совокупность всех перечисленных параметров. Установлено, что определяющим фактором в формировании биопленок являлась фаза роста бактерий, в которой функционировала культура синегнойной палочки перед инокуляцией. При посеве *P. aeruginosa*, пребывающей в стационарной фазе роста, образование биопленок нелинейно зависело от концентрации питательных веществ и общего их количества в объеме культивирования. Линейная зависимость образования биопленок от концентрации питательных веществ в среде культивирования была более выражена при посеве *P. aeruginosa*, находящейся в фазе логарифмического роста. Установлено, что при меньших концентрациях компонентов питательных сред различия в образовании биопленок были более заметны и имели статистическую значимость. Разбавление жидкой питательной среды в 2 раза не влияло на интенсивность формирования пленки, в то время как 4–8-кратное снижение концентрации питательных веществ в объеме культивирования 0,2 мл ингибировало образование биопленок. В объеме среды для культивирования, равном 1,0 мл, формирование биопленок было равномерным, а в объеме 0,2 мл статистически значимо снижалось при разведении питательной среды в 4 и 8 раз. Объем культивирования также имеет важное значение: так, выращенные в 0,2 мл питательной среды культуры при разных концентрациях питательных веществ формировали меньшее количество биопленок, чем микроорганизмы, культивируемые в объеме 1,0 мл. При этом при посеве *P. aeruginosa*, находящихся как в логарифмической, так и стационарной фазах роста, более выраженным было образование биопленок в лунках с большим объемом культивирования.

Ключевые слова: биопленки, бактерии, *P. aeruginosa*, синегнойная палочка, фаза стационарного роста, фаза логарифмического роста

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INTRODUCTION

The ability to form biofilms may be called one of the most important factors that contributes to persistence and protection of microorganisms from the immune response of the macroorganism [1–3]. A biofilm can be defined as a microbial community attached either to the substrate surface or to each other, surrounded by an exopolymer matrix which is the main structural component of the biofilm. Bacterial phenotype observed in the biofilm is modified compared to single, planktonic cells, the growth and expression parameters of specific genes are transformed [4–7].

The bacterial film is a living, constantly updating community of one or several microorganisms, while the surrounding matrix protects them from adverse environmental impact and serves as one of the factors of intercellular communication. The matrix properties determine the relationship between intracellular community and external environment [3, 6, 8, 9].

More than 95% of all microorganisms are found in natural ecosystems in the form of specifically organized biofilms [5]. *Pseudomonas aeruginosa* (blue pus bacillus, *P. aeruginosa*) is one of the microorganisms capable of forming biofilms. It is a ubiquitous infectious agent, which causes a number of opportunistic diseases. *P. aeruginosa* is detected in 20% of septicemia cases, in 70% of mucoviscidosis cases (in sputum), in up to 70% of hospital-acquired pneumonia cases, in 28% of intra-abdominal infection cases. Totally, it accounts for 20–30% among other etiological agents of hospital-acquired infections [10].

P. aeruginosa is characterized by high resistance to antiseptic substances and disinfectants. The microorganism has a wide range of pathogenicity factors, high epidemic

potential and unique adaptive properties, and is capable of reducing effectiveness of the body's immune response [2, 11–14].

The bacteria in biofilms synthesize an alginate (mucoid exopolysaccharide) and form an exopolymeric alginate matrix. Alginate-producing strains are usually detected in chronic infections, for example, the ones associated with mucoviscidosis. The bacterial biofilm also saves the microorganism from body's natural resistance (lymphocytes, phagocytes, natural movements of the ciliated epithelium in the respiratory tract, antibodies, etc.). The role of quorum sensing systems has also been proven in the *P. aeruginosa* biofilm formation [1, 2, 14, 15].

The importance of the study is explained by the fact that *P. aeruginosa* is an extremely frequent pathogen that plays a key role in emergence of many difficult-to-treat infectious diseases, in particular due to its ability to form biofilms.

There are current scientific studies showing correlation between the growth of plankton culture and biofilm formation, however, this correlation is not absolute [2].

A novelty value of this research consists in studying biofilm formation under different conditions of *P. aeruginosa* cultivation, in particular, depending on the concentration of nutrients in the cultivation volume, as well as the culture growth phase used for inoculation.

The decisive factors in biofilm formation during microbiological experiments are: cultural, enzymatic properties, etc. of the studied microbiota; cultivation conditions (temperature, medium composition, concentration of nutrients, etc.); the material on the surface of which the biofilm will be formed, and much more.

The purpose of this research was to study peculiarities of biofilms formed by *P. aeruginosa* bacterial culture under various cultivation conditions.

MATERIALS AND METHODS

Bacteria. P. aeruginosa ATCC 9027 strain was used as an example to analyze how cultivation conditions influence biofilm growth , the strain was taken from the Museum of the Molecular Biology Sector of the Institute of Experimental Veterinary Medicine of Siberia and the Far East, SFSCA RAS.

Cultivation of P. aeruginosa and bacterial biofilms. *P. aeruginosa* ATCC 9027 culture was grown in LB-Luria liquid nutrient medium (0.5 g/L NaCl; HiMedia) after a preliminary series of passages at room temperature in an incubator shaker for 6 and 24 hours. The broth culture was re-inoculated every 24 hours.

Studying how the growth phase of P. aeruginosa broth culture, volume of the nutrient medium in the well and concentration of nutrient components influence biofilm formation. To assess how *P. aeruginosa* growth phase impacts biofilm formation, 6- and 24-hour culture was inoculated into the nutrient medium, i.e. at log and stationary growth phases (which passed a series of passages in this cultivation phase). Then, *P. aeruginosa* broth suspension was diluted to a value of 0.4 (according to the McFarland standard) in the proportion of 100 µl of culture to 10 ml of nutrient medium. The inoculum was prepared by introducing colonies of *P. aeruginosa* ATCC 9027 strain into sterile saline solution, then bringing the density of the microbial suspension up to the specified concentration.

In order to assess, how volume and concentration of nutrients mutually influence the intensity of *P. aeruginosa* biofilm formation, liquid culture media LB-Luria (0.5 g/L NaCl) and Schaedler (HiMedia) used for cultivation were diluted with saline solution to the concentration of 50; 25; 12.5; 6% and introduced into the wells of flat-bottomed polystyrene plates in volumes of 0.2 and 1.0 ml (in four repetitions). Next, a V-shaped-bottom microplate was immersed into the plate wells, and the *P. aeruginosa* broth culture was inoculated. The inoculations were incubated at a temperature of (25.0 ± 0.5) °C for 18 hours.

LB-Luria and Schaedler (HiMedia) nutrient media were used as a negative control without addition of the microbial inoculum; growth of *P. aeruginosa* was controlled in six repetitions.

Biofilm staining method. In order to assess the growth, the biofilms were stained with gentian violet (crystal violet), a dye that binds to cells and the biofilm matrix, according to the existing method [16] in its modified form: after cultivation is completed, the bacteria, that had not attached to the wells surface were carefully washed away three times with deionized water. The biofilms that formed in the microplate wells were dried at room temperature for 2 hours, fixed with alcohol for 40 minutes and stained with 0.05% solution of gentian violet for 40 minutes. The unbound dye was washed away three times with 0.01 M phosphate-buffer saline with pH 7.2 (3 minutes per one wash). Then a microplate with biofilms was immersed into the wells of a polystyrene flat-bottomed microplate containing 200 µl of 96% ethyl alcohol to elute the unbound dye. The quantitative assessment of the formed biofilms was carried out by measuring optical density with a plate spectrophotometer reader Tecan Sunrise (Tecan, Austria) at a wavelength of 450 nm, OD_{450} (by the intensity of alcohol staining with the extracted dye).

Statistical data analysis. The data were processed by methods of variation statistics using the Statistica 13.3 software package. The statistical significance of the differences was assessed using Student's t-test (reliability criterion). Differences at the p level < 0.05 were considered reliable. The correlation between the studied parameters was assessed using the Pearson correlation coefficient.

RESULTS AND DISCUSSION

In this study we proceed from the hypothesis that *P. aeruginosa* synthesizes alginate to store nutrients necessary to ensure energy metabolism in case of nutritional deficiency, thus resulting in the culture growth. Considering this hypothesis, we should expect a non-linear biofilm formation with a decrease in the amount and concentration of nutrients in the culture medium.

According to the results obtained, the intensity of *P. aeruginosa* biofilm formation varies depending on the concentration of nutrients, the volume of nutrient medium in the well and the growth phase of the culture used for inoculation. When concentration of nutrient media components decreased, differences in biofilm formation were more noticeable and were statistically significant (Table 1).

When a *P. aeruginosa* culture, that had been at a stationary growth phase for 48 hours, was used for inoculation, the biofilm formation depended only on concentration of nutrients in the volume of cultivation (Fig. 1). Thus, when the LB medium was twice diluted, the volume of

Table 1
Growth of *P. aeruginosa* ATCC 9027 biofilms under different cultivation conditions, OD_{450}, M ± SD ($n = 4$)

Growth phase of *P. aeruginosa* ATCC 9027 culture/ nutrient medium	Volume of nutrient medium in wells, ml							
	1.0				0.2			
	Concentration of nutrient media, %							
	50	25	12.5	6	50	25	12.5	6
Stationary phase/ LB	0.16 ± 0.00	0.13 ± 0.01	0.12 ± 0.01	0.12 ± 0.02	0.15 ± 0.02	0.16 ± 0.01***	0.15 ± 0.02***	0.08 ± 0.01***
Logarithmic phase/ Schaedler	0.25± 0.02	0.16 ± 0.02	0.16 ± 0.01	0.13 ± 0.01	0.31 ± 0.05*	0.26 ± 0.07**	0.25 ± 0.03**	0.14 ± 0.02
Logarithmic phase/ LB	0.18 ± 0.04	0.17 ± 0.04	0.16 ± 0.03	0.12 ± 0.03	0.17 ± 0.03	0.16 ± 0.02	0.14 ± 0.02**	0.11 ± 0.01

* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

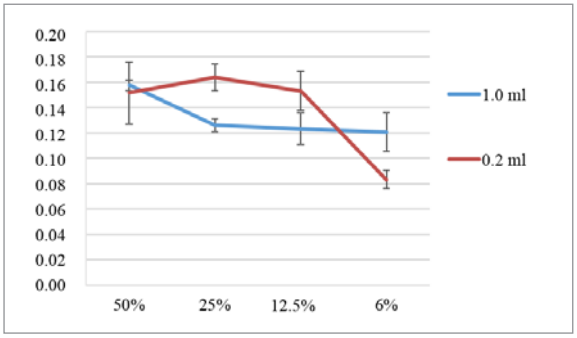

Fig. 1. Growth of P. aeruginosa ATCC 9027 biofilms (inoculation of culture grown up to a stationary phase) in wells containing 0.2 and 1.0 ml of LB medium and different concentrations of nutrients, OD_{450} (M ± SD)

cultivation did not affect the intensity of biofilm growth. After a 4–8-fold dilution of the nutrient medium *P. aeruginosa* formed a biofilm of higher density, when grown in wells with 1.0 ml of broth, than when grown in 0.2 ml of medium ($p = 0.000232$ and 0.000129 respectively). Within this range of nutrient medium concentrations, the intensity of biofilm formation was the same and demonstrated a statistically significant decrease in 0.2 ml cultivation volume in comparison with the culture grown in 1.0 ml of nutrient medium ($p = 0.000825$).

As Figure 2 shows, when *P. aeruginosa* was used for inoculation at a log phase (after a series of passages in this phase of cultivation), the intensity of biofilm formation was also influenced by the concentration of nutrients in the Schaedler medium. It should be noted that 0.2 ml cultivation volume implies a 5-fold difference in the total nutrient content compared to 1.0 ml. Accordingly, all the dependence curves did not intersect, biofilm formation in 0.2 ml cultivation volume was naturally less at all concentrations of nutrients in comparison with cultures grown in 1.0 ml volume.

After a 8-fold dilution of the nutrient medium, there is a sharp decrease in the efficiency of biofilm matrix synthesis, both when cultured in 1.0 ml volume and when grown in 0.2 ml volume. It is confirmed by the fact that the dependence of biofilm formation on the nutrient content is more accurately described by a two-step polynomial function ($R^2 = 0.996–0.979$) than by a linear function ($R^2 = 0.854–0.931$).

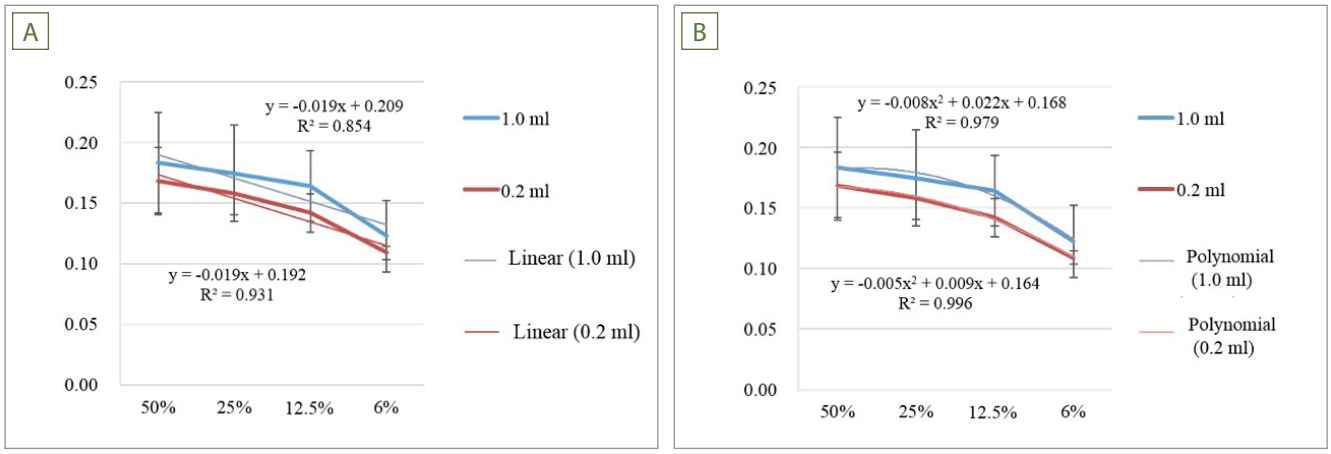

Fig. 2. Growth of P. aeruginosa ATCC 9027 biofilms (inoculation of culture grown up to a log phase) in wells containing 0.2 and 1.0 ml of LB medium and different concentrations of nutrients, OD_{450} (M ± SD): A – linear trend; B – polynomial trend

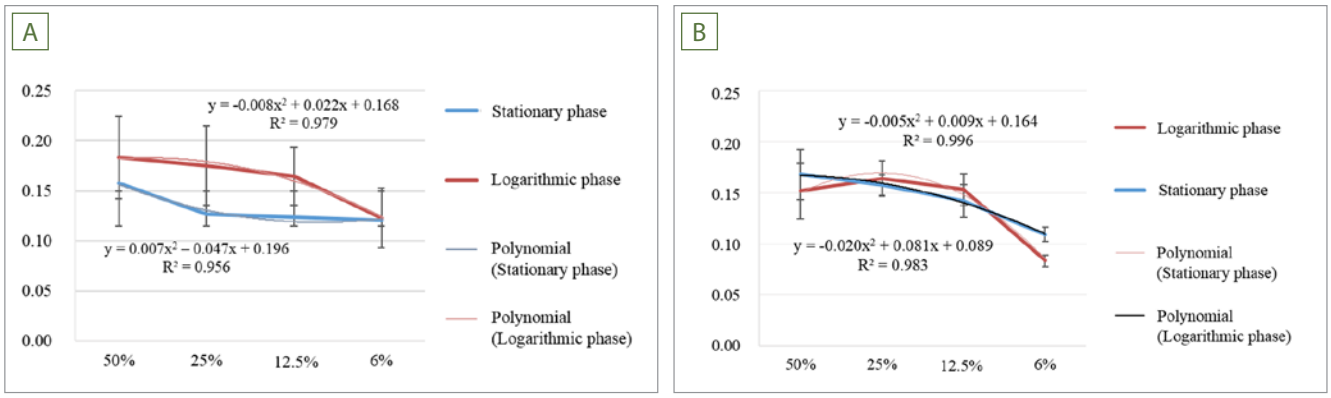

Fig. 3. Growth of P. aeruginosa ATCC 9027 biofilms (inoculation of cultures grown up to log and stationary phases) in wells containing different volumes of LB medium and different concentrations of nutrients, OD_{450} (M ± SD): A – 1.0 ml; B – 0.2 ml

The effect of *P. aeruginosa* growth phase used for cultivation on biofilm formation was most pronounced when cultured in 1.0 ml volume using LB nutrient medium diluted 4 and 8 times ($p = 0.0209$ and 0.0053 respectively). Such dependence was not observed when cultivating these bacteria in 0.2 ml (Fig. 3).

It is natural that a metabolically more active culture at a log phase used the components of the nutrient medium more efficiently. At the same time, the amount of nutrients extracted from 1.0 ml cultivation volume was greater than from 0.2 ml.

The non-linear nature of biofilm formation, in relation to the concentration and total content of nutrients in the culture medium, allows assuming that biofilms are connected with the ability to stockpile and concentrate nutrients. Differences in the intensity of biofilm synthesis associated with the culture growth phase during inoculation, suggest that they are caused not only by genetic variability, but also by the ability to preserve the phenotype due to the epigenetic mechanisms of the quorum sensing in *P. aeruginosa*.

Cultivation of static biofilms in liquid nutrient media resulted in a conclusion that biofilm formation depended on concentration of nutrients, the medium volume and the growth phase of the culture used for cultivation.

As the research demonstrates, a bacterial growth phase, where *P. aeruginosa* culture functions at the moment of inoculation, is a decisive factor in the biofilm formation. When inoculating *P. aeruginosa*, which had been at the stationary growth phase for 48 hours, the biofilm formation showed non-linear dependence on concentration of nutrients and on their total amount in the cultivation volume. Two-fold dilution of the LB nutrient medium did not affect the biofilm growth; however, a 4- to 8-fold decrease in concentration of nutrients in the 1.0 ml cultivation volume stimulated the biofilm formation.

In 1.0 ml cultivation volume, the intensity of biofilm formation was even and did not depend on the degree of the LB medium dilution, and in 0.2 ml cultivation volume it demonstrated a statistically significant decrease, when diluted 4 and 8 times.

The linear dependence of biofilm formation on concentration of nutrients in Schaedler culture medium was more pronounced, when inoculating *P. aeruginosa* at a log phase. The dependence graphs were described by a linear function with a correlation coefficient $R^2 = 0.854–0.931$.

The cultures grown in a smaller cultivation volume (0.2 ml) at different concentrations of nutrients formed biofilms of lower density compared to microorganisms cultivated in 1.0 ml.

Studying the influence of logarithmic and stationary phases of *P. aeruginosa* culture growth on biofilm formation revealed that the biofilms formed more intensively in 1.0 ml cultivation volume. At the same time, a metabolically more active culture inoculated at a log growth phase used the culture medium nutrients more efficiently.

The study of the effect of nutrient concentration and total nutrient content in the cultivation volume on biofilm formation resulted in an assumption that biofilm formation is connected with the function of storing and concentrating nutrient components of the medium. At the same time, it is important to note non-linear nature of the biofilm formation, which confirms the hypothesis that if there is a lack of nutrients promoting its growth, *P. aeruginosa*is able to synthesize alginate which helps to preserve and maintain the concentration of nutrients needed for the population growth. In addition, due to depletion of nutrients alongside with accumulation of metabolic products, the vital activity of all biofilm-forming microorganisms is inhibited, while at low concentrations of nutrients this process naturally decreases.

CONCLUSION

The described study demonstrates that *P. aeruginosa* biofilm formation depends both on the culture growth phase used for inoculation, on the volume of the cultivation well, and on the concentration of nutrients in the cultivation volume.

The research reveals that the culture growth phase, at which microorganisms functioned before being inoculated into liquid nutrient media, was a factor of paramount importance. Determination of optical densities of the studied samples showed, when *P. aeruginosa* was inoculated at a log growth phase, the linear dependence of the biofilm formation on concentration of nutrients in the cultivation volume was more pronounced in comparison with the culture at a stationary growth phase. When *P. aeruginosa* grown up to a stationary phase was used for inoculation,

we observed a uniform growth of biofilms at different concentrations of nutrient components in the medium and cultivation volumes.

Bacterium biofilm formation at a log growth phase in 0.2 ml wells with a decreased concentration of nutrients (50; 25; 12.5 and 6%) in the liquid nutrient Schaedler medium was characterized by the following growth indicators: 0.31 ± 0.05; 0.26 ± 0.07; 0.25 ± 0.03; 0.14 ± 0.02; biofilm growth indicators in 1.0 ml cultivation volume were: 0.25 ± 0.02; 0.16 ± 0.02; 0.16 ± 0.01; 0.13 ± 0.01. When cultivating *P. aeruginosa* in a liquid nutrient LB medium, a pronounced linear dependence of the biofilm formation on the concentration of nutrients was noted. In 0.2 ml wells, the biofilm growth rates were as follows: at a 50% concentration of nutrient media –0.17 ± 0.03; at 25% –0.16 ± 0.02; at 12.5% – 0.14 ± 0.02; at 6% – 0.11 ± 0.01. In 1.0 ml wells, these indicators were: at a 50% medium concentration – 0.18 ± 0.04; at 25% – 0.17 ± 0.04; at 12.5% – 0.16 ± 0.03; at 6% – 0.12 ± 0.03. It was also found that in 1.0 ml cultivation volume the intensity of biofilm formation was uniform, and in 0.2 ml, there was a statistically significant decrease arising from a 4- and 8-fold dilution of the nutrient medium. At the same time, the 4- and 8-fold decrease in the concentration of nutrients in 1.0 ml cultivation volume stimulated the biofilm formation.

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Bacteriology and pathological anatomy of pneumonias in monkeys

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SUMMARY

Data on the etiological structure of potential pneumonia agents in monkeys based on postmortem findings and subsequent bacteriological tests of lung tissues collected from the organ areas showing morphological changes are presented. In the period between 2019 and the first half of 2021, 377 animals died of pneumonia. The highest pneumonia-associated mortality was observed in newborn (0–8-day-old) and baby monkeys under the age of 1 month (161 animals). Polysegmental bronchopneumonia was detected in the dead monkeys in 94.4% of cases, croupous pneumonias accounted for 4.5%. Pneumonia was typically the only disease detected in baby monkeys. The microbial landscape in pneumonia affected monkeys was characterized by a broad diversity: 899 bacteria of different taxonomic groups were isolated from the lung tissues. Staphylococci (23.8%) prevailed among Gram-positive bacteria, *Escherichia coli* (32.1%) – among Gram-negative bacteria. *Streptococcus pneumoniae* made up 0.3%. Based on data from bacteriological tests, the proportion of pneumonias of undetermined etiology was 0.7%. Besides, bacterial associations, two- or three-component ones as a rule, were detected in the tests of lung tissue samples. The most frequent combinations of associative pathogens were the following: *Escherichia coli* + *Proteus* spp. (24.7%), *Staphylococcus aureus* + *Escherichia coli* (19.6%), *Staphylococcus* spp. + *Enterococcus* spp. + *Escherichia coli* (35.5%), *Staphylococcus* spp. + *Escherichia coli* + *Proteus* spp. (21.2%). Almost all the enterobacteria detected have a high associativity coefficient and occur mainly in the form of associations. The analysis of the study results showed that practically any microorganism alone or in combination can cause pneumonia in an animal with a weakened immunity; therefore, the effect of microbiota should not be underestimated. Also, significance of associative microbes in the development of pneumonia in captive monkeys is increasing.

Keywords: monkeys, pneumonia, polysegmental bronchopneumonia, croupous pneumonia, bacterial agents, microbial associations

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Бактериология и патологическая анатомия пневмоний у обезьян

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РЕЗЮМЕ

Приведены данные по этиологической структуре потенциальных возбудителей пневмоний у обезьян согласно данным патолого-анатомической картины с последующими бактериологическими исследованиями тканей легких, взятых из морфологически измененных участков органа. В период с 2019 по 1-е полугодие 2021 г. от пневмоний погибло 377 животных. Наибольшая гибель от пневмоний отмечена у новорожденных (0–8 сут) и детенышей до 1 месяца (161 особь). В 94,4% случаев у погибших обезьян была выявлена полисегментарная бронхопневмония, доля крупозных пневмоний составила 4,5%. У детенышей пневмония чаще являлась единственным заболеванием. Микробный пейзаж при пневмониях обезьян отличался широким разнообразием. Из легочной ткани выделено 899 бактерий разных таксономических групп, из грамположительной микрофлоры преобладали стафилококки (23,8%), из грамотрицательной – *Escherichia coli* (32,1%). Доля *Streptococcus pneumoniae* составила 0,3%. Удельный вес пневмоний неустановленной этиологии, по данным бактериологического исследования, был равен 0,7%. При исследовании образцов легочной ткани также выявлены бактериальные ассоциации, как правило, двух- и трехкомпонентные. Среди патогенов-ассоциантов чаще встречались следующие комбинации: *Escherichia coli* + *Proteus* spp. (24,7%), *Staphylococcus aureus* + *Escherichia coli* (19,6%), *Staphylococcus* spp. + *Enterococcus* spp. + *Escherichia coli* (35,5%), *Staphylococcus* spp. + *Escherichia coli* +

Proteus spp. (21,2%). Практически все выявленные энтеробактерии имеют высокий коэффициент ассоциативности, встречаясь в основном в виде ассоциаций. Анализ результатов исследования показал, что практически любой микроорганизм изолированно или в комбинации может привести к развитию пневмонии при ослаблении иммунитета животного, поэтому нельзя недооценивать влияние микрофлоры. Также возрастает роль микробов-ассоциантов в развитии пневмонии у обезьян, содержащихся в условиях неволи.

Ключевые слова: обезьяны, пневмония, полисегментарная бронхопневмония, крупозная пневмония, бактериальные возбудители, микробные ассоциации

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INTRODUCTION

Respiratory tract pathology is a widespread group of diseases. Pneumonia belonging to this group is an inflammation in lung structures that develops against the backdrop of different factors. In most cases, it occurs as a result of aspiration of opportunistic oropharyngeal microbiota into the lower airways. Therefore, it can be said that pneumonia is a pluricausal disease of mostly bacterial, bacterial-viral or viral etiology. Most common bacterial agents are *Streptococcus pneumoniae*, *Haemophilus influenzae*, *Legionella pneumophila*, *Staphylococcus aureus*, *Klebsiella pneumoniae* and *Mycoplasma pneumoniae*. Along with these pathogenic microorganisms, opportunistic bacteria (enterobacteria, coagulase-negative staphylococci, etc.) today have become more common in the etiological structure of significant agents. However, among many microorganism species colonizing upper airways, only a few can enter the lungs and cause an inflammatory response. Different pathogens can cause pneumonia either alone, or in association with other microorganisms, that is why some authors underscore the polymicrobial nature of this disease [1, 2]. Pneumonias caused by *St. aureus* and *K. pneumoniae* account for the largest percentage of deaths [3]. Given the infectious nature of pneumonia, it is important to study the variety of the disease agents.

Pneumonia affects all animals, but typically cattle, small ruminants, horses, cats, cetaceans. The reasons for the disease occurrence are living conditions (keeping animals in poor conditions), hypothermia, vitamin deficiency, malnutrition and, as a consequence, weakened body defences. The disease most commonly affects newborns and young animals under the age of one year.

Pneumonia is currently one of important problems in primatology, which is not solely due to its high occurrence in monkeys in captivity, but also due to high mortality rates, especially in baby animals under the age of 1 month. Pneumonia kills up to 20–50% of monkeys in the breeding facilities and zoos worldwide [4, 5]. Pneumonia can occur as a primary disease, as well as complicate other diseases. Specific features of pneumonia etiology and pathogenesis in captive monkeys require further detailed examination. There are reports in literature that highlight the close similarity between pneumonia in humans and in monkeys [6].

The aim of the study is to carry out the analysis of pneumonia-associated deaths of monkeys, to identify the range of bacterial pathogens being potential infectious agents.

MATERIALS AND METHODS

In the period from 2019 to the first half of 2021, 866 dead monkeys of both sexes, belonging to sixteen species (Table 1) that had lived in the breeding facility of the FSBSI "Research Institute of Medical Primatology" (FSBSI "RIMP") were necropsied. The age of the dead animals submitted to the necropsy department ranged from 0 days (newborns) to 35 years. Based on the necropsy results, a total of 355 monkeys died in 2019, 316 monkeys – in 2020, and 195 monkeys – in the first half of 2021. Stillbirths, decomposed carcasses and euthanized animals were not taken into consideration in this study.

Based on pathomorphological findings, pneumonia was identified as a principal or secondary diagnosis in 377 animals. Preliminary diagnosis was made based on macroscopic findings from the necropsy. In most cases, it was confirmed, with some additions and refinements, by histology.

Pieces of lungs collected from the organ areas showing morphological changes served as necropsy material used for examination. A part of the material was fixed in the 10% buffered formalin. Hematoxylin-eosin stained histological samples were prepared. The material was examined using a light microscope.

In parallel, bacteriological tests were carried out. For bacterial microbiota detection, lung material was inoculated by replica plating method, as well as to sugar broth, from which the resulting colonies were inoculated to differential diagnostic media after 24 hours. Salt Agar with egg yolk emulsion, Endo Agar, 5% Blood Agar were used for the isolation of different microorganism groups, as previously described [7]. The isolated cultures were identified based on the morphology and biochemical properties.

Table 1
Characteristics of dead monkeys

Monkey species	Age						Total
	under 1 month	1 month –1 year	1–3 years	3–10 years	10–15 years	15 years and over	
Macaca mulatta	29	19	43	87	49	63	290
Macaca fascicularis	48	21	19	70	29	41	228
Macaca nemestrina	5	2	–	5	5	6	23
Chlorocebus sabaeus	6	3	4	10	4	7	34
Papio anubis	18	3	10	27	12	10	80
Papio hamadryas	69	23	16	43	18	22	191
Macaca assamensis	–	–	–	–	1	1	2
Macaca arctoides	–	–	–	–	–	2	2
Macaca maura	–	–	–	–	–	1	1
Cercopithecus mona	–	–	–	–	–	1	1
Lophocebus aterrimus	–	1	–	–	–	–	1
Cebus apella	2	–	2	–	–	1	5
Cebus capucinus	2	–	–	–	–	–	2
Macaca sylvanus	–	–	–	–	–	1	1
Erythrocebus patas	–	–	2	1	–	1	4
Hylobates	–	–	1	–	–	–	1
Total	179	72	97	243	118	157	866

Associativity coefficient (AC) used as a criterion for determination of microbial association frequency and bacterium involvement in them was calculated:

$$AC = \frac{\text{number of associative cultures of a certain species}}{\text{total number of cultures of this species}} \times 100\%.$$

When AC is less than 50% (low criterion), microorganisms mainly occur in monocultures; when AC is 50–79% (medium criterion) – more frequently as associative microorganisms; when AC is 80–100% (high criterion) – mainly in the form of associations.

RESULTS AND DISCUSSION

Out of the total number of monkeys that had died between 2019 and the first half of 2021, 377 (43.5%) were diagnosed with pneumonia based on pathomorphological findings; that was evidenced by various manifestations of inflammation and the location of the organ lesions: lung tissue hardening, darkened areas, catarrhus of tracheal and bronchial mucosa (Table 2). Most frequently, chronic atrophous gastroenterocolitis, in presence of general emaciation and dehydration of the body, was identified as a secondary diagnosis in adult animals affected with pneumonia.

Based on the number of dead monkeys by species, the highest pneumonia-associated mortality percentages were observed in *Papio hamadryas* (28.4%), *Macaca fascicularis* and *Macaca mulatta* (27.3 and 24.9%, respectively) (Table 2). Observations showed that mortality in the animals does not depend on their sex. Specifically, 187 male and 190 female monkeys died during the said period. The analysis of seasonal dynamics demonstrated the absence of a sharp pneumonia-associated mortality increase in monkeys in the course of a year.

According to the data presented in Tables 1 and 3, pneumonia-associated mortality in baby monkeys aged under 1 month was 90% (161 out of 179 animals). Pneumonia also killed more than half of baby monkeys under the age of 1 year (42 out of 72 animals) and 42.3% of adolescent monkeys aged under 3 years (41 out of 97 animals). Pneumonia-associated mortality in young monkeys aged 3 to 10 years (55 out of 243 animals) is more than twice lower than that in adolescent monkeys, and pneumonias are, as a rule, concurrent diseases in such cases. Adult and old animals again demonstrate higher mortality, with pneumonia being the main cause of deaths. Pneumonia-associated mortality regardless of species was found to be the greatest (42.7%) in newborn and baby monkeys aged from several days to 1 month (Table 3). In particular, *Papio hamadryas* demonstrated the highest mortality percentage (37.3%). In the said age group, high mortality was also observed in baby *Macaca fascicularis* (30.4%). In baby *Macaca mulatta*, mortality becomes higher starting from the age of 1 month

to 1 year (23.8%). Pneumonia was typically the only disease detected macroscopically in baby monkeys under the age of 1 year. Mortality rates in the adult and old monkeys of the genus *Macaca* (*Macaca mulatta* and *Macaca fascicularis*) are approximately the same, whereas mortality in baboons decreases with the increase of years.

In most cases, the dead monkeys were diagnosed with bilateral polysegmental bronchopneumonia (94.4%). Lobar pneumonia was detected in 4.5% of cases.

Necropsy revealed that mucous membranes of trachea and large bronchi were swollen, engorged and with multiple petechias (Fig. 1).

Microscopic examination revealed the presence of such cellular elements as neutrophilic leukocytes, lymphocytes, squamous alveolar cells and erythrocytes in different proportions (Fig. 2).

Microfocal and confluent serous pneumonia prevailed. Loose lying or phagocytized diplococci were typically detected in exudates. Bronchial disorders manifested themselves as desquamative or ulcerative bronchitis. Mixed microbiota represented by cocci was detected in the bronchial lumen. The mentioned lung lesions often occurred in combination with multifocal atelectasis. The following was detected in lobar pneumonia cases: gross lesions of one or several lung lobes, fibrinous exudate in the alveoli, fibrin deposition on the pleura (pleuropneumonia), exudate consisted predominantly of fibrin. Pneumonias in monkeys under the age of 6 months were systemic, affecting the entire lung, accompanied by bacteriaemia and purulent inflammation of meninges. Lobar pneumonia and bronchopneumonia are classical anatomical categories of bacterial pneumonia with morphological features depending on infectious agent species, and bacteriological tests play a critical role in identifying the etiology of infectious processes in such cases.

Table 2
Number of monkeys (by species) that died of pneumonias of different etiology between 2019 and the first half of 2021

Monkey species	Number (%) of animals			Total
	2019	2020	the first half of 2021	
Macaca mulatta	42 (44.7)	30 (31.9)	22 (23.4)	94 (24.9)
Macaca fascicularis	42 (40.8)	43 (41.7)	18 (17.5)	103 (27.3)
Macaca nemestrina	6 (35.3)	9 (52.9)	2 (11.8)	17 (4.5)
Chlorocebus sabaeus	5	5	–	10 (2.7)
Papio anubis	19 (54.2)	8 (22.9)	8 (22.9)	35 (9.3)
Papio hamadryas	42 (39.3)	40 (37.4)	25 (23.3)	107 (28.4)
Macaca assamensis	2	–	–	2 (0.5)
Macaca sylvanus	1	–	–	1 (0.3)
Erythrocebus patas	2	–	–	2 (0.5)
Cebus apella	1	1	2	4 (1.1)
Cebus capucinus	–	2	–	2 (0.5)
Total	162 (43.0)	138 (36.6)	77 (20.4)	377

A total of 899 microorganism cultures were detected in bacteriological tests of monkey lung samples, with Gram-positive microbiota accounting for 45.1% (405 cultures) and Gram-negative microbiota – for 54.9% (494 cultures). There was no growth on nutrient media in 0.7% of cases (7 lung samples). The analysis of microbial

Table 3
Age structure of monkeys that died of pneumonias

Monkey species	Number (%) of animals aged					
	under 1 month	1 month – 1 year	1–3 years	3–10 years	10–15 years	15 years and over
Macaca mulatta	24 (14.9)	10 (23.8)	15 (36.6)	20 (36.4)	13 (34.2)	12 (30.0)
Macaca fascicularis	49 (30.4)	12 (28.6)	8 (19.5)	14 (25.5)	8 (21.1)	12 (30.0)
Macaca nemestrina	5 (3.1)	1 (2.4)	–	5 (9.1)	3 (7.9)	3 (7.5)
Chlorocebus sabaeus	5 (3.1)	–	–	2 (3.6)	–	3 (7.5)
Papio anubis	15 (9.3)	–	4 (9.8)	5 (9.1)	8 (21.1)	3 (7.5)
Papio hamadryas	60 (37.3)	18 (42.8)	11 (26.8)	8 (14.5)	5 (13.1)	5 (12.5)
Macaca assamensis	–	–	–	1 (1.8)	–	1 (2.5)
Macaca sylvanus	–	–	–	–	1 (2.6)	–
Erythrocebus patas	–	–	2 (4.9)	–	–	–
Cebus apella	1 (0.6)	1 (2.4)	1 (2.4)	–	–	1 (2.5)
Cebus capucinus	2 (1.3)	–	–	–	–	–
Total	161 (42.7%)	42 (11.1%)	41 (10.9%)	55 (14.6%)	38 (10.1%)	40 (10.6%)

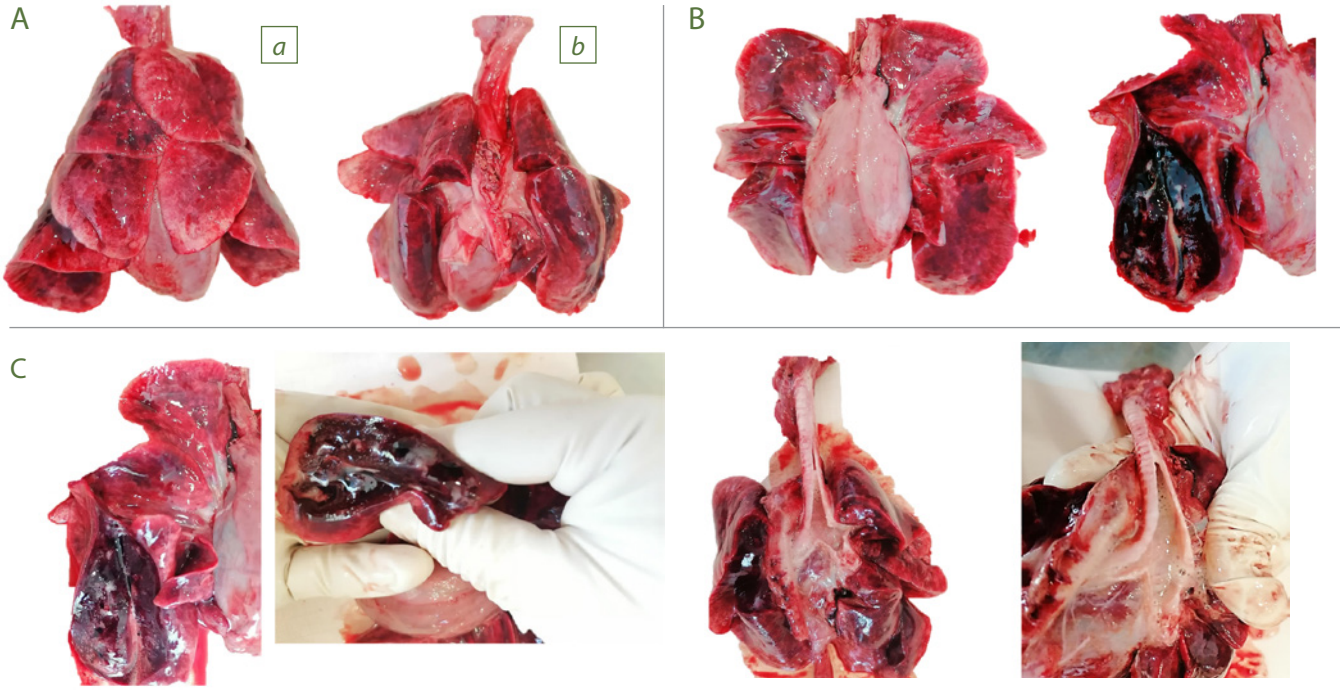

Fig. 1. Bilateral polysegmental bronchopneumonia (gross lung lesions) in a 4-year-old male Macaca mulatta
A – ventral (a) and dorsal (b) surfaces of lungs;
B – lower lobe of the right lung;
C – pulmonary alveolar edema

landscape showed that the representatives of the family *Enterobacteriaceae* (54.1%) were detected most frequently, Gram-negative non-fermenting bacteria were detected in 0.2%, and *Pseudomonas aeruginosa* – in 0.7% of cases. Among coccal flora, staphylococci (23.8%) occurred most frequently, *St. aureus* accounted for 16.8%, *Enterococcus* spp. – for 15.2%, *St. pneumoniae* bacteria were detected in as little as 0.3% of cases. Among enterobacteria, *Escherichia coli* were detected most frequently (32.1%). The percentages for other bacteria were low (Fig. 3).

Enterobacteria of the genus *Klebsiella* (2.6%) isolated from pneumonia cases were represented by three species: *K. pneumoniae* (10 isolates), *K. oxytoca* (11 isolates), *K. ozaenae* (2 isolates). Among the members of the genus *Enterobacter* (2.5%), the most frequently detected ones were *E. cloacae* (7 isolates) and *E. aerogenes* (6 isolates); *E. gergoviae* (3 isolates), *E. agglomerans* (4 isolates) were detected less frequently. The bacteria of the genus *Citrobacter* were detected in 1.2% of cases, with the number of *C. freundii* isolates being 8, *C. diversus* – 2 and *C. farmeri* – 1. The representatives of the genus *Providencia* were detected in 0.7% of cases: *P. stuartii* (5 isolates), *P. rettgeri* (1 isolate). Other enterobacteria were detected in individual cases: *Erwinia* spp. (2 isolates), *Hafnia alvei* (2 isolates), *Serratia* spp. (1 isolate). The proportion of detected *Bacillus* spp. was 0.6%, that of other unidentified Gram-positive rod-shaped bacteria – 4.3%. As Table 4 shows, the isolated microorganisms were represented

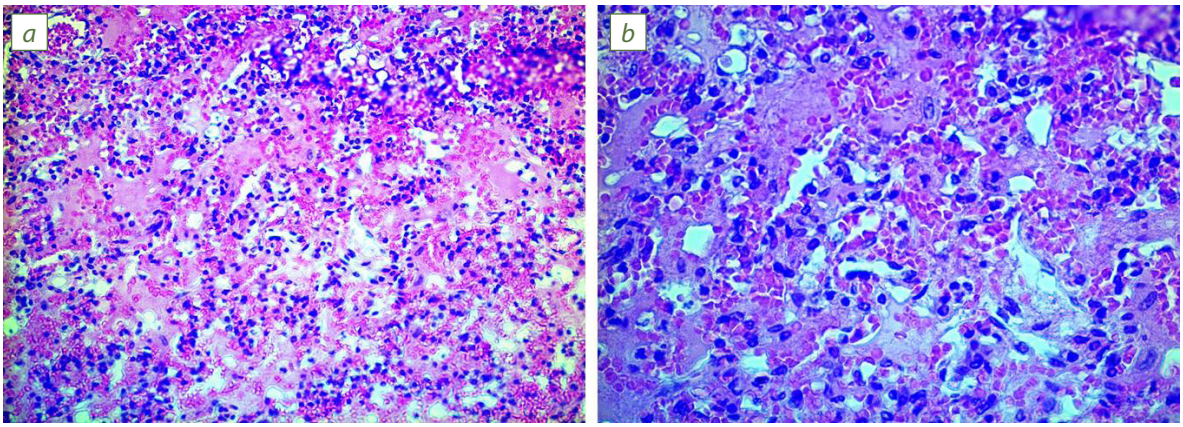

Fig. 2. Bilateral polysegmental bronchopneumonia (microscopic lung lesions) in a 4-year-old male Macaca mulatta. Hematoxylin and eosin staining, 100× (a) and 200× (b) magnification

by monocultures in 13.4% of cases, while other bacteria were part of associations (86.6%) that comprised from 2 to 6 microorganisms.

As a result of the tests performed, an increase in the number of associations was detected; in particular, in 2019, 142 associations were isolated, and in the first half of 2021 – as many as 138 microbial associations. Two-component associations (58.4%) had a leadership position.

Also, three-, four-, five-component associations (29.8, 10.3, 1.2%, respectively) and one six-component association (0.3%) were detected. Table 5 shows the number of the most frequently registered bacterial associations. As can be seen, the most frequent combination detected among two-component associations was *E. coli* + *Proteus* spp. (24.7%), among three-component associations – that of *Staphylococcus* spp. + *Enterococcus* spp. + *E. coli* (35.5%). Other combinations were present in individual cases.

When determining the frequency of microorganism occurrence as part of associations, associativity coefficient (AC) was calculated. Bacteria isolated from pneumonia cases were characterized by medium and high associativity coefficients. *E. coli* demonstrated a medium AC (58%), others had a high AC ranging from 67% for *Ps. aeruginosa* to 100% for *Providencia* ssp. and *Citrobacter* spp.

The shortcoming of the study was the absence of *Mycoplasma pneumoniae* and *Chlamydia pneumoniae* diagnosis. However, based on the previously conducted studies, these pathogens can be present in the lung tissues of pneumonia-affected monkeys [8]. According to the data on file from the Laboratory of Infectious Virology of the FSBSI "RIMP", no respiratory viruses were detected in pneumonia-affected monkeys during the period from 2019 to June 2021. The molecular genetic studies of *St. aureus* carried out earlier make it possible to speak about their high pathogenicity and consider them as etiologically significant agents of pneumonias in monkeys [9]. The isolation of such enterobacteria as *Enterobacter* spp., *Citrobacter* spp., *Proteus* spp., *Morganella morganii*, *Providencia* spp., *Hafnia alvei*, *Serratia* spp., *Erwinia* spp., as well as of *E. coli* in most cases from lung tissues is indicative of *post mortem* contamination of the tested material, rather than of the etiological significance of these bacteria. Nevertheless, the analysis of the data obtained shows that practically any microorganism alone or in combination can cause pneumonia in an animal with a weakened immunity; in view of this, the effect of microbiota must not be underestimated, and it can be said that significance of associative microbes in the development of pneumonia is increasing [2].

CONCLUSIONS

Thus, the study performed yielded the following conclusions:

1. Lower respiratory tract diseases (pneumonias) of different etiology are often reported in captive non-human primates of various species. In different years, there has been some disease dynamics in relation to sex and age, most likely associated with weather conditions and the number density of monkeys in enclosures and cages.

2. In baby monkeys, pneumonia progresses rapidly and is fatal.

3. Pneumonia is often identified as a concurrent disease in adult monkeys with gastro-intestinal disorders.

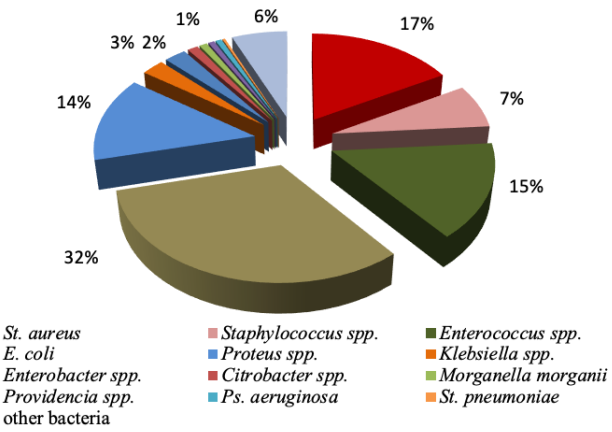

Fig. 3. Structure of bacterial cultures isolated from pneumonia-affected monkeys

Table 4
Frequency of isolation of monocultures and microbial associations from the lungs of pneumonia-affected monkeys

Microorganism	Total	Monoinfection, abs. number (%)	Associations, abs. number (%)
Staphylococcus spp.	63	10 (15.9)	53 (84.1)
St. aureus	151	4 (2.7)	147 (97.3)
E. coli	288	57 (19.8)	231 (80.2)
Proteus spp.	122	5 (4.1)	117 (95.9)
Ps. aeruginosa	6	2 (33.3)	4 (66.7)
Bacillus spp.	6	2 (33.3)	4 (66.7)
Enterococcus spp.	137	40 (29.2)	97 (70.8)

Table 5
Variants of most common combinations of microorganisms in the lungs of pneumonia-affected monkeys

Two- and three-component associations	Abs. number, (%)
E. coli + *Proteus* spp.	48 (24.7)
St. aureus + *E. coli*	38 (19.6)
St. aureus + *Enterococcus* spp.	21 (10.8)
E. coli + *Enterococcus* spp.	19 (9.8)
St. aureus + *Proteus* spp.	16 (8.3)
E. coli + *Enterobacter* spp.	10 (5.2)
Other combinations	42 (21.6)
Total	194
Staphylococcus spp. + *Enterococcus* spp. + *E. coli*	35 (35.4)
Staphylococcus spp. + *E. coli* + *Proteus* spp.	21 (21.2)
Other combinations	43 (43.4)
Total	99

4. Lobar, polysegmental processes (94.4%) prevail in the structure of lung lesions in pneumonia-affected monkeys.

5. The following classical signs of pneumonia were detected *post mortem* in dead primates: the presence of exudate, colour change (inflammatory hyperemia) and hardening of affected area. All lesions were advanced and serve as an illustrative example of classical disease progression in cases diagnosed with bilateral polysegmental bronchopneumonia and lobar pneumonia.

6. As a result of bacteriological tests of lung tissues from the monkeys that had died of pneumonias, different bacterial pathogens and their combinations were detected. The percentage of *St. aureus* within the microbial landscape structure was 16.8%, that of *Enterococcus* spp. – 15.2%, of *St. pneumoniae* – 0.3%. Among enterobacteria, *E. coli* (32.1%) were detected most frequently. The most frequently reported microbial associations were the combinations of *E. coli* + *Proteus* spp. (24.7%), *Staphylococcus* spp. + *Enterococcus* spp. + *E. coli* (35.4%). Almost all the enterobacteria detected were identified as associative ones.

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Immunodiffusion assay as a method of bovine leukosis post-mortem diagnosis

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SUMMARY

New method of post-mortem diagnosis of bovine leukosis is proposed and it involves use of agar gel immunodiffusion assay. The proposed method allows for the detection of antibodies against bovine leukemia virus (BLV) antigen located in the muscle and tissue fluids (plasma and lymph) of meat and offal. Post-mortem sampling was performed by dragging sterile cotton swabs across different parts of carcass and organs of both animals known to be seronegative and animals not tested alive. The collected samples and accompanied documents were submitted to the laboratory. 0.5–0.7 mL of isotonic solution (0.85% sodium chloride solution) were added to the tube with the swabs and the tube was left for 3–5 hours at 18–26 °C until homogenous substance formation. The tube was occasionally shaken so that BLV antibodies moved to the normal saline solution for further immunodiffusion assay. The assay results were visually recorded by detection of precipitation lines. Testing of 175 samples collected from animals not serologically tested for bovine leukosis before slaughter demonstrated five positive results (2.9%). Immunodiffusion assay of the tissue (lymphatic) fluid swabs collected from 148 animals, declared BLV seronegative alive in the veterinary certificates, demonstrated negative results. Therefore, along with autopsy, histological, molecular and genetic methods the immunodiffusion assay can be one of the tools for post-mortem diagnosis of bovine leukosis.

Keywords: bovine leukosis, post-mortem diagnosis, immunodiffusion assay, serology, homogenous substance

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Применение реакции иммунодиффузии как один из способов послеубойной диагностики лейкоза крупного рогатого скота

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РЕЗЮМЕ

Предложен новый способ послеубойной диагностики лейкоза крупного рогатого скота с применением реакции иммунной диффузии в геле агара. Предлагаемый метод позволяет выявлять антитела к антигену вируса лейкоза крупного рогатого скота, находящиеся в мышечно-тканевой жидкости (в плазме и лимфе) мяса и субпродуктов. Послеубойный отбор проб производили стерильным ватным тампоном путем смыва из разных частей туши и органов как заведомо серонегативных, так и не исследованных прижизненно животных. Полученные образцы биологического материала доставляли с сопроводительными документами в лабораторию. В пробирку со смывом добавляли от 0,5 до 0,7 мл изотонического раствора (0,85%-й раствор хлорида натрия) и оставляли на 3–5 ч для перехода в однородную субстанцию, выдерживали при температуре 18–26 °С и периодически встряхивали, чтобы антитела к вирусу лейкоза крупного рогатого скота со смыва переходили в физиологический раствор для дальнейшей постановки реакции иммунодиффузии. Учет результатов при проведении реакции проводили визуально путем выявления линий преципитации. При исследовании 175 образцов биологического материала от животных, не исследованных прижизненно на лейкоз крупного рогатого скота, серологическим методом положительный на лейкоз результат был получен в 5 (2,9%) случаях. При постановке реакции иммунодиффузии с пробами смывов с тканевой (лимфатической) жидкости, отобранными от 148 животных, которые на основании ветеринарных справок были прижизненно серонегативными к вирусу лейкоза крупного рогатого скота, получили

отрицательные результаты. Таким образом, применение реакции иммунодиффузии может стать одним из способов послеубойной диагностики лейкоза крупного рогатого скота наряду с патолого-анатомическими, гистологическими, молекулярно-генетическими методами.

Ключевые слова: лейкоз крупного рогатого скота, послеубойная диагностика, реакция иммунодиффузии, серология, однородная субстанция

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INTRODUCTION

Bovine leucosis is induced by bovine leukemia virus (BLV) belonging to *Retroviridae* family. According to the International Committee on Taxonomy of Viruses (ICTV)[1], from 2020 *Retroviridae* family includes 68 species, 11 genera and two sub-families: *Orthoretrovirinae* and *Spumaretrovirinae*. Sub-family *Orthoretrovirinae* includes six genera: *Alpharetroviris*, *Betaretrovirus*, *Deltaretrovirus*, *Epsilonretrovirus*, *Gammaretrovirus*, *Lentivirus*; sub-family *Spumaretrovirinae* – five genera: *Bovispumavirus*, *Equispumavirus*, *Felispumavirus*, *Prosimiispumavirus*, *Simiispumavirus*. *Bovine leukemia virus*, or BLV, belongs to genus *Deltaretrovirus*, which, in addition to BLV, includes three more species: primate T-lymphotropic viruses (HTLV-I, HTLV-II, HTLV-III) [1–3]. BLV affects hematopoietic and lymphoid tissues of animals and involves bone marrow, spleen, lymph nodes, etc. into the pathologic process. At late disease stage, other organs are also affected (stomach, liver, intestines, lungs, etc.) due to proliferation and malignant degeneration of blast cells [4–6].

Veterinary laboratories make lifetime diagnosis of bovine leucosis using different methods, such as serological ones involving enzyme-linked immunosorbent assay (ELISA) and agar gel immunodiffusion (AGID), molecular-genetic tools involving polymerase-chain reaction (PCR), haemotological, clinical and cytomorphologic methods [7–9].

Post-mortem diagnosis of bovine leucosis is made on the basis of *post-mortem* examination and histological tests of fallen or emergently slaughtered animals. At necropsy, *post-mortem* lesions of organs and tissues are recorded depending on the leucosis lesion progression and nature. In case of lymphocytic leukemia, the spleen and lymph nodes are enlarged and bone marrow metaplasia is reported. On late stages, lymphoid hyperplasia is also observed in other organs. In case of monocytic leukemia, there are no *post-mortem* lesions on early stages, but on the later ones the lymph nodes get enlarged and often fused. In case of acute hemocytoblastic leukemia, spleen and lymph nodes are enlarged is size and weight. In case of myeloid bovine leukemia, the *post-mortem* lesions are reported in lymph nodes, spleen and liver; myelocyte foci are reported as well.

[1] https://talk.ictvonline.org.

Autopsy examinations do not always demonstrate lesions in animal organs, especially on early stages of the disease. Sometimes bovine leucosis cannot be successfully differentiated from many other diseases (actinomycosis, tuberculosis, paratuberculosis, brucellosis, etc.). In case the disease pathological pattern is not clear enough, the diagnosis should be confirmed by laboratory tools involving production of histological preparations of animal organs and muscles and their examination using light and electronic microscopes [10–12].

PCR-based molecular-genetic method is equally important for the *post-mortem* diagnosis of bovine leucosis. This method can be applied both for lifetime and *post-mortem* diagnosis of bovine leucosis. PCR can detect DNA of bovine leucosis provirus in animal blood or muscle tissue samples.

Due to the complexity of the above-mentioned tests (*post-mortem*, histological, molecular-genetic), new AGID tool can be relevant for the *post-mortem* diagnosis of bovine leucosis thus facilitating the efforts of the veterinarians. Therefore, the work was aimed at the use of AGID-based serological method for *post-mortem* diagnosis of bovine leucosis.

MATERIALS AND METHODS

The material used for bovine leucosis *post-mortem* diagnosis included 323 samples collected from bovine carcasses and offal (liver, spleen, kidney, etc.) on the market No. 2 in Makhachkala, Republic of Dagestan. Among these, 148 samples were collected from animals, which were declared seronegative when alive by the veterinary certificates; and 175 samples were collected from the animals not subjected to *ante-mortem* serological tests for BLV. Since BLV antibodies are detected in muscle tissue fluid (plasma and lymph) using AGID, the samples were collected as swabs taken by sterile cotton from the carcass surfaces as well as from the muscle tissues incised by sterile scalpel. The collected samples were delivered to the laboratory for serological testing. The samples were transported in sterile tubes with designation of the number, animal species, date, time and place of swab collection, etc.

Post-mortem AGID tests of animal carcasses and offal were performed in the laboratory using the test-kit for

bovine leucosis serological diagnosis manufactured by FKP "Kursk Biofactory" (Russia).

Samples (swabs) were collected from the carcasses and internal organs according to the "Rules for ante-mortem examination of slaughter animals and post-mortem inspection of meat and meat products" [13], and the serological tests were carried out using "Methodical guidance for bovine leucosis diagnosis" [14].

RESULTS AND DISCUSSION

All veterinary and sanitary requirements applicable to biomaterial collection from the carcasses and offal for further *post-mortem* examination were strictly followed. The collected swabs were transferred to the numbered sterile tubes and delivered to the Laboratory of farm animal infectious pathologies in the Caspian Zonal Veterinary Research Institution. The samples were accompanied with relevant documents. Before AGID, depending on the size of the swab 0.5–0.7 mL of isotonic solution (0.85% sodium chloride solution) were once added to the tubes with the swabs and the tubes were left for 3–5 hours until homogenous substance was formed. After that the tubes were kept at room temperature (18–26 °C) and occasionally shaken (2–3 time) so that antibodies contained in the swabs collected from BL-infected carcasses could transfer to the saline solution.

AGID was carried out according to the following scheme: 0.04–0.06 mL of the tested substance were inoculated in the wells punched in the agar gel. The tested substance was inoculated in wells No. 1, 3, 4 and 6 using automated pipette (dosing device); BLV antigen was inoculated in central well No. 7 and opposite peripheral wells No. 2 and 5 were inoculated with precipitating serum (Fig.). Then, the Petri dishes were incubated in thermostat at 20–26 °C, and the results were visually recorded in 48 hours.

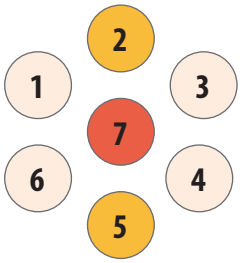
Ante-mortem BL diagnosis using AGID involves detection of the anti-BLV-specific precipitating antibodies in animal sera.

To assess AGID as a *post-mortem* BL diagnosis tool, laboratory tests of 148 isotonic solution-diffused tissue (lymphatic) fluid samples collected from carcasses and offal of animals confirmed to be BL-negative by AGID as well as 175 samples randomly collected from animals not subjected to *ante-mortem* BL testing were performed (see Table)

The Table demonstrates that all 148 samples from animals confirmed BLV seronegative during lifetime demonstrated negative results during *post-mortem* BL diagnosis using AGID.

AGID-tests of 175 biological samples collected from animals not subjected to *ante-mortem* testing for BL demonstrated 5 (2.9%) positive results on Day 1 and 2 post sample collection. On Day 5 post swab collection, the antibodies were detected only in 4 (2.3%) of the tested samples. The key task of the *post-mortem* tests performed at different time-points post sample collection (Day 1, 2, 5) was to identify decrease (maintenance) of the level of antibodies against BLV antigen in muscle tissue fluids (lymph and plasma) using AGID.

Serological tests of the samples collected from the surfaces as well as from the incised muscle tissues of the animal carcasses and offal demonstrated that AGID is an inexpensive and user-friendly tool for *post-mortem* diagnosis of bovine leucosis.



1, 3, 4, 6 – tested substance (solution with the swab);
2, 5 – specific precipitating serum;
7 – freeze-dried BLV antigen

Fig. ID assay of test substance for post-mortem diagnosis of bovine leucosis

Table
***Ante-mortem* and *post-mortem* BL diagnosis using serological method (AGID)**

Ante-mortem bovine leucosis diagnosis	Total AGID-tested, animals	*Post-mortem* diagnosis using AGID, terms of tests		
		Day 1	Day 2	Day 5
BLV-negative in AGID	148	–	–	–
Serological (other) diagnostic tests for bovine leucosis were not carried out	175	5	5	4
Total	323	5 (2.9%)	5 (2.9%)	4 (2.3%)

CONCLUSION

The foregoing prompts the conclusion that immunodiffusion test can be used for *post-mortem* bovine leucosis diagnosis during meat inspection along with other recognized methods – pathological and histological tests as well as molecular-genetic PCR.

Therefore, *post-mortem* serological testing of carcasses and offal for bovine leucosis demonstrated AGID suitability as one of the test-systems for detection of antibodies to BLV antigen in tissue fluids (plasma and lymph) and offal of animals during *post-mortem* examination [15].

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Comparative analysis of intestinal microbiome of local cattle and Aberdeen Angus cattle imported to Kazakhstan

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SUMMARY

Animal microbiome plays a significant role in all the vital body processes. Studying the microbiome is essential for gaining a detailed insight into the interactions among microorganisms inhabiting a certain organ and their relationship with macroorganism cells. Evaluating the state of animal microbial community and its function can provide an invaluable assistance in seeking new strategies to improve feed efficiency and maintain cattle health. The aim of the study was to compare the taxonomic structure of the intestinal microbiome of Aberdeen Angus cattle imported to Kazakhstan with that of local breed cows using next generation sequencing technology. The tests of fecal samples allowed for determination of the complete microbial composition of animal intestinal contents, while leaving out the preliminary stage of microbiological cultivation using nutrient media. The results of 16S metagenomic analysis showed that *Firmicutes* and *Proteobacteria* were predominant bacterial taxons at the phylum level in the intestinal microbiome in both groups of animals, with their numbers being roughly the same. At the bacterial family level, the number of *Clostridiaceae* was a little higher in Aberdeen Angus cows (19.7%) than in the local breed cattle (15.4%). The representatives of the families *Bacteroidaceae*, *Peptococcaceae*, *Ruminococcaceae* and *Coriobacteriaceae* prevailed in the gut microbial community of local cattle. These microorganisms are involved in the synthesis of vitamins, they stimulate the immune function of the body, normalize digestion, improve nutrient utilization and thus contribute to body weight gain in animals. Small numbers (0.5%) of bacteria of the family *Prevotellaceae* were detected only in the local breed cows demonstrating high body weight gain. The microbiome of the local cattle was characterized by a considerable diversity at the genus level: the total number of taxons amounted to 65, whereas in Aberdeen Angus cattle it was 40. It was found that the intestinal microbiome of local breed cattle includes less methanogens and more acetogens.

Keywords: microbiome, cattle, Aberdeen Angus, next generation sequencing

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Сравнительная характеристика кишечного микробиома местного крупного рогатого скота и скота абердин-ангусской породы, импортированного в Казахстан

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РЕЗЮМЕ

Микробиом животных играет существенную роль во всех жизненно важных процессах организма. Его изучение необходимо для детального понимания процессов, происходящих между микроорганизмами, населяющими определенный орган, и их взаимосвязи с клетками макроорганизма. Оценка состояния микробного сообщества животных и его функции может оказать неоценимую помощь в поиске новых стратегий повышения эффективности кормления и сохранения здоровья крупного рогатого скота. Целью исследования было сравнение таксономической структуры микробиома кишечника крупного рогатого скота абердин-ангусской породы, импортированного в Казахстан, и коров местных пород с помощью технологии секвенирования нового поколения. Был определен полный микробный состав содержимого кишечника животных при исследовании образцов экскрементов без предварительной стадии микробиологического культивирования на питательных средах. Результаты 16S метагеномного анализа показали, что доминирующими бактериальными таксонами в микробиоме кишечника животных обеих групп на уровне типа были *Firmicutes* и *Proteobacteria* примерно в одинаковом количестве. На уровне бактериальных семейств численность представителей *Clostridiaceae* была немного больше у коров абердин-ангусской породы (19,7%), чем у скота местной породы (15,4%). Представители семейств *Bacteroidaceae*, *Peptococcaceae*, *Ruminococcaceae* и *Coriobacteriaceae* преобладали в микробном сообществе кишечника местного скота. Данные микроорганизмы участвуют в синтезе витаминов, стимулируют иммунную функцию организма, нормализуют пищеварение, увеличивают усвояемость питательных веществ и, как следствие, повышают привесы у животных. Бактерии семейства *Prevotellaceae* были выявлены в небольшом количестве (0,5%) только у коров местной породы, которые имели высокие привесы. На уровне рода значительное разнообразие наблюдали в микробиоме местного скота: всего 65 таксонов против 40 у абердин-ангусов. Установлено, что в кишечном микробиоме крупного рогатого скота местных пород содержится меньшее количество метаногенов и большее количество ацетогенов.

Ключевые слова: микробиом, крупный рогатый скот, абердин-ангусская порода, секвенирование нового поколения

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INTRODUCTION

Ruminants, in particular cattle and small ruminants, serve as an important source of food for humans. The Aberdeen Angus is considered to be the world's top marbled beef cattle breed with delicious and incredibly succulent meat. Aberdeen Angus cows are low-maintenance cattle that grow and gain meat mass rapidly. Daily weight gain in steers can be from 1 to 5 kg. This breed has become very popular due to fast aging and high quality meat; therefore, a large number of Aberdeen Angus cattle has been imported to the Republic of Kazakhstan in recent years. However, the process of the cattle adaptation under the conditions of Kazakhstan has not been explored; in particular, it is not yet known what effect the local climate and diet fed to the animals have on their body and productivity.

Microbiome is an important constituent of living organisms that has effect on immunity, productivity and vital functions in animals. The intestinal microbiome of cows, which comprises bacteria, archaea, protists and fungi, is responsible for production of various enzymes required for plant fibre degradation into volatile fatty acids and microbial crude protein. Studying the composition of the microbial community involved in rumen microbial metabolism is of great interest for the development of new strategies to improve feed efficiency and maintain cattle health [1]. Microbiome also includes methanogenic archaea that determine the amount of methane emitted by livestock, which is one of the current environmental concerns.

Most microbes cannot be cultured *in vitro* and grown using laboratory nutrient media. The cultivation of anaerobes is rather complicated due to the slow microbial growth, the need for restricting the access of oxygen and other requirements regarding cultivation parameters [2]. Methagenomic analysis allows for microbial community description using highly efficient new generation sequencing (NGS) technology based on DNA identification, while leaving out microbiological cultivation stage. Sequencing of hypervariable regions of highly conserved

and universal 16S rRNA genes is widely used for bacterial community and archaeon characterization [3, 4].

The aim of the study was to compare the taxonomic structure of the intestinal microbiome of Aberdeen Angus cattle with that of local breeds with a view to assessing its effect on cattle immunity, productivity and methane production under the conditions of the Republic of Kazakhstan.

MATERIALS AND METHODS

Fecal samples were collected in triplicate from the rectum of three seventh-generation Aberdeen Angus cattle and three local breed cows on the neighbouring farms located in the Almaty Oblast. All the intestinal content samples were immediately frozen in dry ice and delivered to the laboratory, where they were kept at minus 80 °C until DNA extraction.

16S metagenomic analysis was performed using the MiSeq™ sequencer (Illumina, USA) and MiSeq™ reagent Kit V3 (300 cycle) (Illumina, USA).

Fecal microbial DNA was extracted using PureLink™ Microbiome DNA Purification Kit following the manufacture's procedure (Invitrogen, USA). DNA concentration was measured with the Qubit™ 2.0 fluorometer (Invitrogen, USA).

Gene libraries were prepared according to the 16S Metagenomic Sequencing Library Preparation protocol (Part # 15044223 Rev. A, Illumina, USA). Variable V3 and V4 regions of 16S rRNA gene were amplified using the following universal primers appended with Illumina adapters: forward primer – 5′-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGWGCAG-3′ and reverse primer – 5′-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC-3′ [5]. The reaction mixture included: 2.5 µl of DNA template, 5 µl of each primer with a concentration of 1 µM, 12.5 µl 2× KAPA HiFi HotStart ReadyMix (Kapa Biosystems, Inc., USA). PCR amplification was performed in the Eppendorf Mastercycler pro S thermal cycler (Eppendorf, Germany) using the following programme: 95 °C for 3 minutes; 25 cycles: 95 °C for 30 seconds, 55 °C for 30 seconds, 72 °C for 30 seconds, one cycle at 72 °C for 5 minutes. PCR product concentration and size were measured with Bioanalyzer 2100 (Agilent, USA).

Then Nextera XT Index primer adapters (Illumina, USA) were added to each sample by amplification in the following reaction mixture: 12.5 µl of KAPA HiFi HotStart ReadyMix, 5 µl of each index primer, 10 µl of water and 5 µl of each PCR product. Amplification was performed using the following programme: 95 °C for 3 minutes; 8 cycles: 95 °C for 30 seconds, 55 °C for 30 seconds, 72 °C for 30 seconds, one cycle at 72 °C for 5 minutes.

Before and after adding the indices, PCR product was purified using Agencourt AMPure PCR purification kit (Beckman Coulter, Inc., USA).

The prepared libraries were normalized to a concentration of 4 nM and pooled. The libraries were combined with the sequencing control reagent MiSeq™ PhiX Control Kit (Illumina, USA), loaded into the sequencing kit cartridge, then the cartridge and the flow cell were loaded into the device. Sequencing reaction was carried out using MiSeq™ Control Software v2.6. Pooled libraries were sequenced in the MiSeq sequencer (Illumina, USA) using MiSeq reagent Kit V3 (300 cycle) (Illumina, USA).

Data were analyzed and processed using MiSeq™ Reporter Software (Illumina, USA). The taxonomic classification was carried out by means of comparison with 16S rRNA gene data from the international database Greengenes Database Lawrence Berkeley National Laboratory (LBNL, USA) (http://greengenes.lbl.gov).

RESULTS AND DISCUSSION

The taxonomic identification of all the bacteria present in the intestinal microbiome was carried out based on the following taxonomic ranks: kingdom, phylum, class, order, family, genus and species.

As Figure 1*a* illustrates, most of the operational taxonomic units detected in Aberdeen Angus cattle feces were identified as belonging to the following bacterial phyla: *Firmicutes* (55%), *Proteobacteria* (16.8%), *Actinobacteria* (9.1%), *Bacteroidetes* (5.1%), *Euryarchaeota* (3.4%), *Verrucomicrobia* (1.6%). The following bacterial phyla

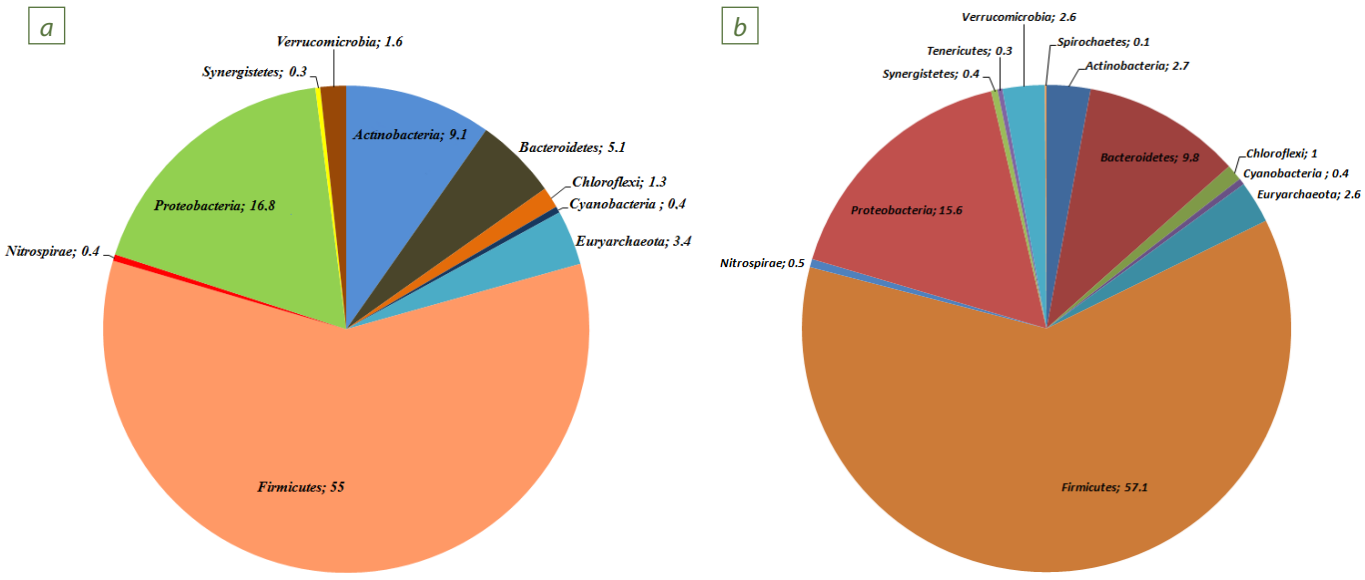

Fig. 1. Relative abundance (% of the total number) of major types of bacteria detected in the intestinal microbiome of cattle: a – Aberdeen Angus and b – local breeds of Kazakhstan

prevailed in the local breeds: *Firmicutes* (57.1%), *Proteobacteria* (15.6%), *Bacteroidetes* (9.8%), *Actinobacteria* (2.7%), *Euryarchaeota* (2.6%) and *Verrucomicrobia* (2.6%) (Fig. 1*b*).

According to S. Y. Mao et al. [6] and R. W. Li et al. [7], excessive grain feeding reduces the number of *Bacteroidetes* bacteria in the microbial community of cattle digestive tract, and this in turn results in the intensive propagation and increase in the number of opportunistic microorganisms of the phyla *Firmicutes* and *Proteobacteria.* High fecal starch concentration is associated with an increase in the number of *Bacteroidetes* and a decrease in the number of *Firmicutes* [8]. In our case, Aberdeen Angus cows were fed with forage (silage, haylage) without any concentrated feed added. Local breed cattle were grazed on the pastures. It was found that the bacteria of the phyla *Firmicutes* and *Proteobacteria* prevailed over *Bacteroidetes* in the gut microbiome composition of both groups of cattle. *Bacteroidetes* are key polysaccharide degrading bacteria, as regards the complex polysaccharides of plant cell walls, due to the presence of glycoside hydrolase and polysaccharide lyase. Since the enzymes synthesized by bacteria in the body of cattle contribute to fibre breakdown and digestion, the reduction of *Bacteroidetes* proportion can lead to digestive disorders in animals [7, 8]. The increase in the number of microorganisms of the phyla *Firmicutes*, *Proteobacteria* and *Cyanobacteria* involved in digestion and utilization of feed nutrients is associated with a pronounced increase in animal body weight gain rates [9–11].

Data presented in Figure 2 show that the following bacterial families prevailed in Aberdeen Angus cows: *Clostridiaceae* (19.7%), *Lachnospiraceae* (7.1%), *Enterobacteriaceae* (6.7%), *Planococcaceae* (5.9%), *Moraxellaceae* (4.1%), *Ruminococcaceae* (3.4%), *Methanobacteriaceae* (3.3%), *Coriobacteriaceae* (2.5%), *Peptostreptococcaceae* (2.1%), *Corynebacteriaceae* (1.8%), *Porphyromonadaceae* and *Erysipelotrichaceae* (1.0% each). The following bacterial families prevailed in the local cows: *Clostridiaceae* (15.4%), *Lachnospiraceae* (8.5%), *Moraxellaceae* (7.0%), *Planococcaceae* (6.8%), *Ruminococcaceae* (5.5%), *Enterobacteriaceae* and *Coriobacteriaceae* (3.3% each), *Methanobacteriaceae* and *Veillonellaceae* (2.5% each), *Bacteroidaceae* (2.2%), *Porphyromonadaceae* (1.6%).

According to the available literature data [12–14], the bacteria of the families *Lachnospiraceae*, *Enterobacteriaceae*, *Turicibacteraceae* and *Bifidobacteriaceae* are predominant among gut microbiota of the cattle fed with grains as a major part of their diet, whereas *Bacteroidaceae*, *Porphyromonadaceae*, *Paraprevotellaceae* are more frequently detected in the animals that feed on grass. However, the data from this study do not support the mentioned statement. The large numbers of microorganisms of the families *Bacteroidaceae* and *Peptococcaceae* that are involved in the sythesis of vitamins, normalize digestion, stimulate the immune function of the body and suppress pathogen microbes were detected in the local cattle. It is believed that the number of *Prevotellaceae* in the cows fed with unprocessed grain is 10 times higher than that in the animals fed with forage only. The metagenomic analysis carried out within this study revealed that the number of microorganisms of this family detected in the local breed cattle was low (0.5%).

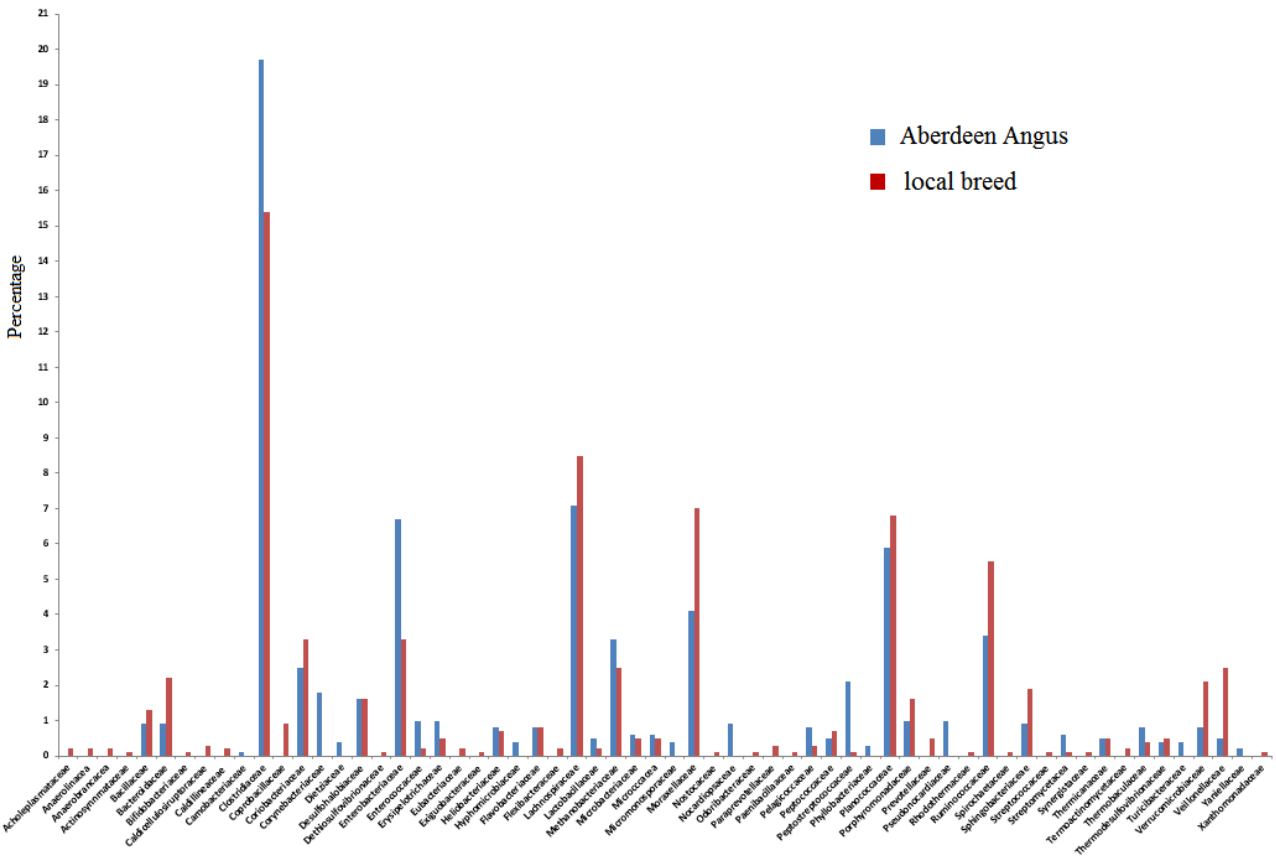

Fig. 2. Gut microbial community profile (bacterial family level) of Aberdeen Angus and local breed cattle of Kazakhtan

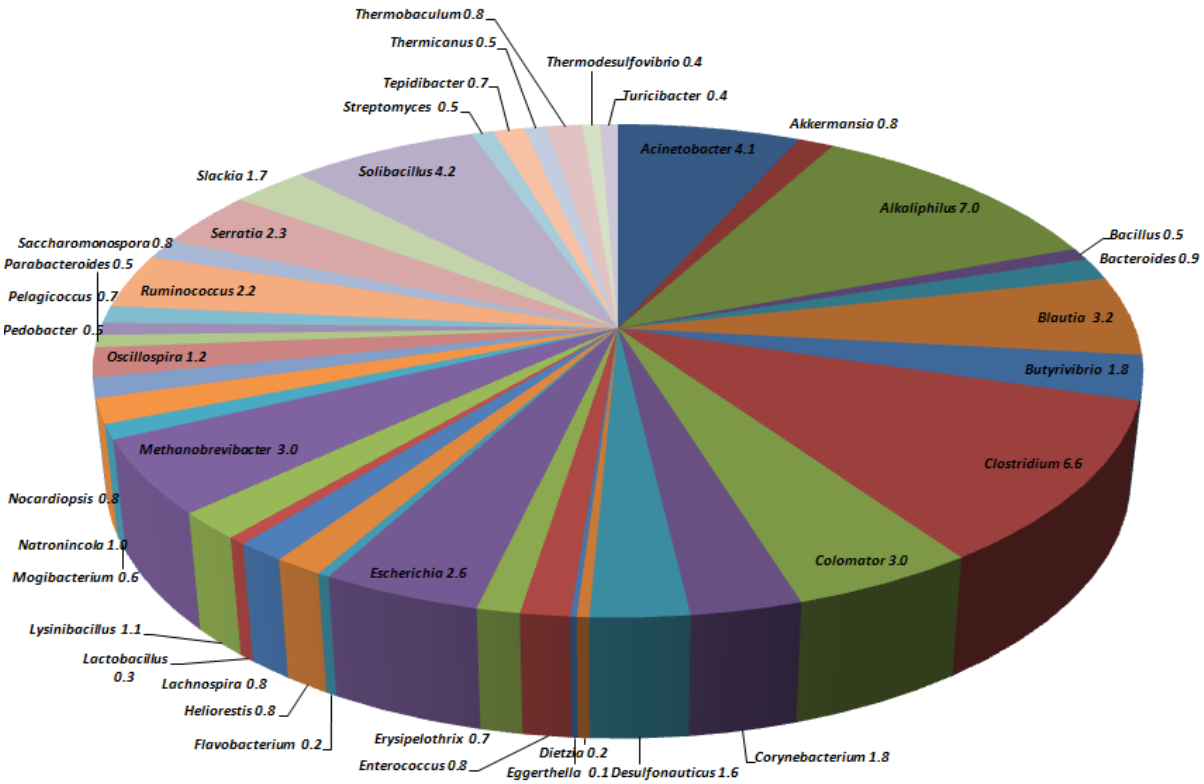

Fig. 3. Gut microbial community profile (bacterial genus level) in Aberdeen Angus cattle

Ruminococcaceae is the most abundant family of microorganisms within the rectal microbiome of animals feeding on grass. This group of bacteria uses dietary fibre as their energy source. The increased numbers of *Ruminococcaceae* and *Lachnospiraceae* in the gut microbial community are indicative of a more complete fermentation of dietary fibre, starch and improved utilization of nutrients. These taxons are also represented by acetogens that use hydrogen as their energy source. An increase in the number of these microorganisms is associated with the reduction of methane production, as was observed during the experiment in the local cattle. In grain fed cows, the bacteria of the family *Ruminococcaceae* transform primary bile acids into secondary bile acids, thus promoting normal digestion [15–17].

High numbers of *Clostridiaceae* are observed in the jejunum in the animals fed with concentrated feeds and in weaned calves. Most of *Clostridiaceae* are commensal bacteria involved in carbohydrate and protein digestion. Some *Clostridiaceae*, such as *Clostridium perfringens*, cause a number of human and animal infectious diseases [16, 17]. The bacteria of the family *Coriobacteriaceae* are capable of modulating lipid metabolism in animals; therefore, their large numbers are detected in the steers that demonstrate high body weight gain [16].

Shabat S. K. et al. found that the microorganisms of the family *Lachnospiraceae* prevailed within the intestinal microbiome of the milking cows with the lowest feed efficiency [18]. F. Li et al. also observed a larger proportion of *Lachnospiraceae* in the cattle with low feed efficiency [19]. However, these data are not consistent with the studies carried out by P. R. Myer et al., who proved that the number of the representatives of the family *Lachnospiraceae* is higher in the microbiota composition in the steers showing the highest weight gain [20]. An increase in the number of *Lachnospiraceae* contributes to a more intensive breakdown of feed components by bacterial enzymes in the caecum, thus leading to an increased synthesis of volatile fatty acids and an increase in the amount of nutrients. Many representatives of the family *Lachnospiraceae* produce butyrate, a microbial metabolite serving as the source of energy for intestinal epithelial cells [3, 14, 15]. The undertaken study revealed that the number of *Lachnospiraceae* detected in the local cattle was higher than that in Aberdeen Angus cattle. As P. R. Myer et al. noted, the number of microbes of the family *Erysipelotrichaceae* was higher in the caecum of steers demonstrating the highest body weight gain and the lowest daily average feed intake [20]. *Erysipelotrichaceae* bacteria are involved in lipid metabolism, and a decrease in their number promotes an increase in intestinal permeability and inflammation development [15]. These microorganisms were detected in all the Aberdeen Angus cows and one local breed cow. The family *Enterobacteriaceae* includes, along with harmless symbionts, certain familiar pathogens [16]. Our study revealed the presence of the bacteria of the genus *Serratia* in Aberdeen Angus cattle (2.3% of cases) and in the local breed cows (0.9%); the bacteria of the genus *Escherichia* were detected in all the Aberdeen Angus cows (2.6%) and in one local breed cow (1.2%). It is known that the microorganisms of the genus *Escherichia* inhibit gut transit and intestinal motility [21], the representatives of the genera *Escherichia* and *Streptococcus* produce toxins [20]. The study showed that *Escherichia albertii,* which possesses the *eae* gene as distinct from *Escherichia coli,* was the most abundant species of microorganisms among those

detected in all the Aberdeen Angus cows and in two local cattle [22]. *Escherichia coli* was detected only in one head of local cattle (0.3%). In the tests of fecal samples from local cattle, *Clostridiaceae* bacterium genome was detected in one animal (1.3%). The proportion of *Escherichia coli* was significantly higher in case of inflammation of intestine, resulting in disbiosis. An increase in the number of pathogenic *Escherichia coli* and *Clostridium perfringens* populations was observed in the rumen and hind gut of cows, in the diet of which grain prevails [7].

Genus-level profile of bacteria detected in Aberdeen Angus and local breed cows is displayed in Figures 3 and 4.

Figure 3 shows that the bacterial genera *Alkaliphilus* (7.0%), *Clostridium* (6.6%), *Acinetobacter* (4.1%), *Solibacillus* (4.2%), *Blautia* (3.2%), *Colomator* and *Methanobrevibacter* (3.0%), *Serratia* (2.3%), *Ruminococcus* (2.2%), *Escherichia* (2.6%) were the major rectal microbiome taxons in Aberdeen Angus cattle. The following bacterial genera prevailed in the local breed cattle: *Clostridium* (7.5%), *Acinetobacter* (7.0%), *Blautia* (3.7%), *Solibacillus* (2.7%), *Alkaliphilus* and *Colomator* (2.6%), *Ruminococcus* and *Oscillospira* (2.5%), *Escherichia* (1.2%) (Fig. 4).

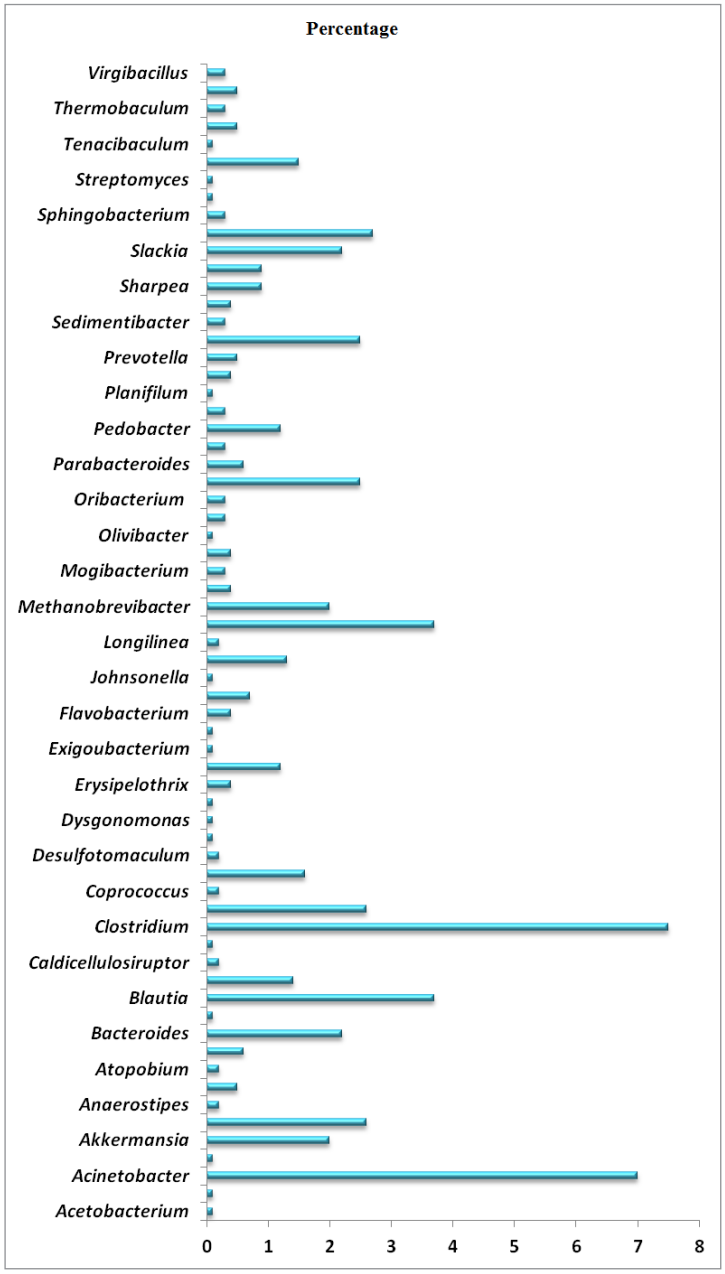

Fig. 4. Gut microbial community profile (bacterial genus level) in local breed cattle

Methane produced by methanogenic bacteria residing in the rumen of cattle is one of the air pollution sources [23]. The bacteria of the genus *Methanobrevibacter* were detected in all the Aberdeen Angus cows and in two local cattle, the bacteria of the genus *Methanosphaera* were detected only in one local breed cow.

The representatives of the genus *Lactobacillus* were detected only in two Aberdeen Angus cattle. *Lactobacillus* species produce lactic acid (lactate) as the major final product of carbohydrate metabolism and are involved in the biological transformation of bile acids. In the course of the study, the bacteria of the genera *Lactobacillus*, *Streptococcus* and *Sharpea* were detected in one local breed cow, *Selenomonas* – in two local cows. The microorganisms of the genus *Ruminococcus* are involved in polysaccharide degradation [15, 17]. As literature data show, *Ruminococcus* species are more abundant in the animals fed with grain, whereas *Solibacillus* and *Acinetobacter* are more frequently detected in the cows fed with grass [3, 16], and this is consistent with the results of our experiments.

It is known that the microorganisms of the genera *Streptococcus* and *Bifidobacterium* prevail in the gut microbial community in the cattle fed a high grain diet and in case of rumen acidosis. These bacteria produce lactic acid as a result of starch fermentation in the rumen [14, 16]. The members of the genus *Bifidobacterium* demonstrate antimicrobial activity and produce acetate [14, 15]. Our study revealed that the representatives of these genera were present only in one local breed cow.

The microorganisms of the genera *Butyrivibrio* and *Blautia* are frequently found in feed-efficient steers [11]. *Butyrivibrio* species degrade pectin, phenylalanine, tyrosine and tryptophane [12, 15]. The bacteria of the genus *Blautia* are characterized by hydrogen and carbon dioxide utilization and the ability to produce acetate (acetic acid) during complex carbohydrate degradation. The bacteria of the genus *Akkermansia* produce fatty acids, such as acetate, propionate and butyrate. The number of *Akkermansia* decreases in case of inflammatory intestinal disorders [15, 20]. The bacteria of the genus *Lysinibacillus* use oxygen in the process of sugar and simple carbohydrate metabolism. *Alcaliphilus peptidifermentans*, a peptide fermenting and iron Fe (III) reducing microorganism [24], was detected in three Aberdeen Angus and two local cattle.

In the animals fed with concentrated feeds, the representatives of the genus *Prevotella* were found to be the key propionate and succinate producing bacteria [7]. *Prevotella* species are involved in polysaccharide and protein breakdown, they are found in the rumen and capable of growing effectively in the acidic environment at a pH of 5.1 [12, 14]. The number of these bacteria increases in case of methanogenesis inhibition. Besides, *Prevotella* species can degrade pectin and produce methanol in the rumen [12, 23]. The bacteria of this genus were detected only in two local breed cows.

CONCLUSION

Thus, during the studies, the taxonomic structure was determined and comparative analysis was carried out with regard to the intestinal bacterial microbiome of imported and local breed cattle.

The fact that the members of the phylum *Euryarchaeota* prevail in Aberdeen Angus cattle as compared to the local cattle is indicative of increased methane production. In particular, the methanogenic bacteria of the genera *Methanobrevibacter* and *Methanosphaera* were more frequently detected in Aberdeen Angus cattle. The bacteria of the families *Lachnospiraceae* and *Blautia* prevailed in the gut microbiome of the local cattle, and this is indicative of their advantage over Aberdeen Angus cattle, since these families are acetogenic bacteria. The microorganisms of the family *Prevotellaceae*, also being acetogenic, were detected in the local cattle only.

The opportunistic microorganisms of the genus *Serratia* were detected in all the tested imported cows. *Escherichia coli* and *Serratia* were detected in one local breed cow; *Clostridium perfringens* were detected in another cow.

The representatives of the families *Bacteroidaceae* and *Peptococcaceae* prevailed in the gut microbial community of the local cattle. The bacteria of the family *Prevotellaceae* were detected only in the local breed cows demonstrating high body weight gain. The representatives of the families *Ruminococcaceae* and *Coriobacteriaceae* prevailed in the local cattle. These microorganisms normalize digestion, improve nutrient utilization, thus leading to an increase in animal body weight gain. The bacteria of the genus *Bifidobacterium* were detected only in one head of local cattle.

Thus, the results of the study showed that the intestinal microbiome of the local cattle includes a smaller number of methanogens and widely represented acetogens; besides, several pathogens, apparently associated with grazing, were detected.

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Epizootic situation on sheep pox and goat pox in Tajikistan in 2000–2021

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SUMMARY

Modeling of potential nosoareals of sheep pox and goat pox that was performed in 2016 revealed a possible trend for aggravation of epizootic situation intensity with regard to these diseases up to 2020, but taking into account the cyclical fluctuations of the situation, it was assumed that the intensity level might reduce in 2017–2020. The highest frequency of reporting these nosounits was characteristic of regions with a higher probability of sheep pox and goat pox occurrence. The retrospective analysis was used to determine the structure, dynamics and properties of the epizootic process of these infectious diseases in the Republic of Tajikistan in 2000–2021. The main causes of sheep pox and goat pox outbreaks in the country were identified. The obtained results confirming the methodological validity of the epizootic situation analysis carried out in 2016 and correctness of the developed models of potential sheep pox and goat pox nosoareals, can become the basis for epizootological prediction of infectious disease risk when the pathogen interacts with susceptible animal population in specific climatic, socio-economic, organizational and managemental conditions. Based on systemic epidemiological analysis of the structure and dynamics of nosoareals, as well as risk assessment of sheep pox and goat pox entry, emergence and distribution, monitoring and establishment of infected and endemic zones, the features and patterns of distribution and occurrence of these diseases' epizootic process were defined, which confirms the need for a systematic approach to epidemiological surveillance of highly dangerous and economically significant infectious diseases having a trend for transboundary spread, which will facilitate the solution of the problem regarding identification of possible threats and the implementation of veterinary and sanitary measures in case of disease occurrence in the territory of any country to ensure animal disease freedom.

Keywords: epizootic situation, sheep pox, goat pox, indicators of epizootic situation intensity, modeling of potential nosoareal

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Эпизоотическая ситуация по оспе овец и оспе коз в Таджикистане в 2000–2021 гг.

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РЕЗЮМЕ

Выполненное в 2016 г. моделирование потенциальных нозоареалов оспы овец и оспы коз до 2020 г. выявило возможность тренда на усугубление напряженности эпизоотической обстановки по этим болезням, но с учетом циклических колебаний ситуации допускалась возможность снижения напряженности в 2017–2020 гг. Наибольшая частота регистрации этих нозоединиц была характерна для регионов более высокой вероятности возникновения оспы овец и оспы коз. При ретроспективном анализе определены структура, динамика и особенности эпизоотического процесса данных инфекционных болезней в Республике Таджикистан в 2000–2021 гг. Выявлены основные причины возникновения вспышек оспы овец и оспы коз в стране. Полученные результаты, подтверждая методическую обоснованность осуществленного в 2016 г. анализа эпизоотической ситуации и верность разработанных моделей потенциальных нозоареалов оспы овец и оспы коз, могут стать основой для эпизоотологического прогнозирования риска возникновения инфекционных болезней при взаимодействии патогена с популяцией восприимчивых животных в конкретных климатогеографических, социально-экономических и организационно-хозяйственных условиях. При системном эпизоотологическом анализе структуры и динамики нозоареалов, результатов оценки риска заноса, возникновения и распространения, мониторинга и определения неблагополучных и эндемичных по оспе овец и оспе коз зон определены особен-

ности и закономерности распространения и проявления эпизоотического процесса при этих заболеваниях, что подтверждает необходимость системного подхода к эпизоотологическому надзору за особо опасными и экономически значимыми инфекционными болезнями, имеющими тенденцию к трансграничному распространению, который будет способствовать решению проблемы определения возможных угроз и реализации ветеринарно-санитарных мероприятий в случае возникновения заболеваний на территории любой страны для сохранения эпизоотологического благополучия.

Ключевые слова: эпизоотическая обстановка, оспа овец, оспа коз, показатели напряженности эпизоотической ситуации, моделирование потенциального нозоареала

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INTRODUCTION

Complex global epizootic situation on a number of highly dangerous small ruminant (ovine and caprine) diseases of viral etiology, including sheep pox (SP) and goat pox (GP), determines relevance of the epizootological monitoring for risk assessment of introduction, emergence and spread of these infections, causing significant economic losses in sheep and goat industry as a result of animal mortality and reduction in productivity and costs of veterinary, sanitary, preventive and quarantine measures.

To determine the structure, dynamics and features of the SP and GP epizootic process in Tajikistan in 2000–2021, a retrospective analysis was carried out taking into account the results obtained by A. V. Kneize et al. when modeling potential nosoareals of these diseases up to 2020 [1]. The development of effective anti-epidemic measures is based on epizootological forecasting, the purpose of which is to determine the risk of an infectious disease when a pathogen interacts with susceptible animal population in specific natural, socio-economic, organizational and managemental conditions [2, 3].

MATERIALS AND METHODS

The research was carried out in accordance with the "Methodical guidelines for conducting epizootological monitoring of exotic highly dangerous and little-known animal diseases" [4] and the provisions of the OIE Terrestrial Animal Health Code [5].

The analysis was based on the data available on the websites of the World Organization for Animal Health (OIE, http://www.oie.int), the Food and Agriculture Organization of the United Nations (FAO, http://www.fao.org), the Rosselkhoznadzor (https://fsvps.gov.ru /), the reports of the State Veterinary Surveillance Service of the Ministry of Agriculture of the Republic of Tajikistan, the Food Safety Committee under the Government of the Republic of Tajikistan and its structural divisions (the National Center for Food Safety Diagnosis (NCFSD) and the Republican Anti-Epizootic Center).

The epizootological method of SP and GP monitoring included: a) comparative descriptive techniques and methods; b) analytical techniques for the formulation and statistical verification of hypotheses, multidimensional statistical analysis and modeling, field and computational experiments; c) methods of synthesis and classification of the obtained knowledge on patterns of epizootic situation development, reasons and conditions of infectious disease emergence, substantiation of methods and means of the epizootic situation control [4].

To form samples characterizing the SP and GP reporting process and outbreaks in 2000–2021, the indicators of the epizootic situation intensity were calculated: the stationary rate (SR is the ratio of disease reporting period duration (years) in the country to duration of the period under investigation (years), measured in fractions of a unit from 0 to 1.0) and the incidence rate (IR is the frequency of reporting new outbreaks in livestock population in an affected country within a year). The SP and GP incidence rate and the stationary rate were evaluated in fractions relative to unity: 0–0.2 – low; 0.2–0.4 – below average; 0.4–0.6 – average; 0.6–0.8 – above average; 0.8–1.0 – high [4, 6].

RESULTS AND DISCUSSION

Small ruminant pox studied by T. Ya. Vannovskii [7], N. V. Likhachev et al. [8], U. G. Kadyrov and Yu. F. Borisovich [9], I. T. Sattorov et al. [10] and others [11, 12] was first officially reported in Tajikistan in 1949. Over one hundred of small ruminant pox-infected settlements were identified in 1951–1952 in Leninabad (now Sughd), Kulyab (now Khatlon) Regions and Regions of Republican Subordination (RRS). Notably goats (80–90%) were the most seriously affected, 18–28% of animals died [10].

Over a 35-year period under study from 1961 to 2000, excluding 1971–1972, 1987–1988 and 1991 (no data

available), the SP infection lasted 22 years in Tajikistan (66 outbreaks were recorded with SR of 0.63 – above average; the average number of outbreaks during the disease registration periods (MNODRP) – 3; the average number of outbreaks during the observation periods (MNOOP) – 1.89). The corresponding indicators in other Central Asian Republics were as follows: Kazakhstan – 25 years (186 outbreaks, SR 0.71 – above average; MNODRP – 7.44; MNOOP – 5.31), Kyrgyzstan – 19 years (129 outbreaks, SR 0.54 – average; MNODRP – 6.79; MNOOP – 3.69), Turkmenistan – 14 years (35 outbreaks, SR 0.4 – average; MNODRP – 2.5; MNOOP – 1) and Uzbekistan – 10 years (45 outbreaks, SR 0.29 – below average; MNODRP – 4.5; MNOOP – 1.29). In Western Asia there were also SP outbreaks being recorded in Azerbaijan for 15 years (45 outbreaks, SR 0.43 – average; MNODRP – 3; MNOOP – 1.29), Armenia – 10 years (64 outbreaks, SR 0.29 – below average; MNODRP – 6.4; MNOOP – 1.83). During this period 3 outbreaks (3-year infection period) were identified in Ukraine with SR 0.09 – low; MNODRP – 1; MNOOP – 0.09, and in Russia the period of infection lasted 16 years when 142 outbreaks were reported (SR 0.46 – average; MNODRP – 8.88; MNOOP – 4.06). From 1961 to 2000 SP was being detected for 34 years in the CIS countries (Kazakhstan, Kyrgyzstan, Tajikistan, Turkmenistan, Uzbekistan, Russia and Ukraine) (SR 0.97; MNODRP – 21.03; MNOOP – 20.43), when 715 outbreaks were registered (Table 1). It should be noted that Kyrgyzstan and Uzbekistan border on the Republic of Tajikistan (RT) and are its trading partners, as well as have partnership links with Kazakhstan, Turkmenistan, Ukraine and Russia, and the fact that Kazakhstan, Kyrgyzstan, Tajikistan and Russia are permanently SP and GP affected determines the severity of the problem.

The largest number of SP outbreaks in the RT was observed in 1964 and 1965 (9 for each year), the smallest – in 1967, 1978, 1983, 1986, 1993 and 1998 (1 for each year), and in 1970, 1973, 1977, 1980–1982, 1984, 1985, 1989, 1990, 1994, 1997 and 1999 the disease was not registered.

According to the OIE data [5], small ruminant pox was detected in all regions (Gorno-Badakhshan Autonomous Region, Sughd and Khatlon Regions, RRS) of Tajikistan in 1992, 1993, 1996–1998, 2000–2003, 2005–2010.

It was reported [10] that only SP was registered in the RT in the period of 1961–2000, and though there are no data on morbidity and mortality available, it is assumed that on average there were about 160 diseased animals per outbreak, and the epizootic cycle in the long-term dynamics was 5 and 8 years. There is also an opinion [13] that both SP and GP outbreaks occurred in Tajikistan (1992–1998) and in other CIS countries: Kyrgyzstan (1994–1997), Azerbaijan (1995), Turkmenistan (1995), Uzbekistan (1995), Kazakhstan (1995–1997).

Considering the risk of SP and GP entry, emergence and spread in the territories bordering on Afghanistan (Sh. Shokhin, M. S. A. Hamadoni, Farkhor, Panj, Jayhun, Shahrituz, Qubodiyon, Nosiri Khusrav) and adjacent to them (Mu'minobod, Vose', Danghara, Vakhsh, J. Balkhi, Kushoniyon, Khuroson) Districts, as well as in Kulyab city in the Khatlon Region, monitoring of morbidity and vaccine prophylaxis efficacy was carried out since 2003 (since 2011 – on the entire RT territory). The epizootic threat of SP and GP entry, emergence and spread in all regions of the country is posed by joint grazing on the pastures of flocks belonging to various farms located in these areas. It should be noted that five valley rivers including the Kofarnihon and the Vakhsh flow in the Khatlon Region and form the Amu Darya, which, as well as the upper source of the Panj River, is a boundary with permanently SP and GP-infected Afghanistan, determining the possibility of a sub-regional risk of these diseases' introduction, occurrence and spread.

The epizootological forecast for the Russian Federation and neighboring states for the period from 2016

Table 1
Data on sheep pox epizootic situation in CIS countries in 1961–2000

Part of the world	Subregion	Country	Total		SR	MNODRP	MNOOP
			outbreaks	infection period (years)			
Asia	Western Asia	Azerbaijan	45	15	0.429	3.000	1.286
		Armenia	64	10	0.286	6.400	1.829
	Central Asia	Kazakhstan	186	25	0.714	7.440	5.314
		Kyrgyzstan	129	19	0.543	6.789	3.686
		Tajikistan	66	22	0.629	3.000	1.886
		Turkmenistan	35	14	0.400	2.500	1.000
		Uzbekistan	45	10	0.286	4.500	1.286
Europe	Eastern Europe	Ukraine	3	3	0.086	1.000	0.086
		Russia	142	16	0.457	8.875	4.057
		Total	715	34			
		Average			0.971	21.030	20.430

to 2020 showed that the highest probability of SP and GP occurrence was determined for the North Caucasian, Southern and Crimean Districts, and the greatest hazard of the disease introduction came from the countries bordering on Russia or having close economic ties with it, such as Tajikistan, Kyrgyzstan, Kazakhstan, Uzbekistan, Turkmenistan, Armenia, Georgia, Azerbaijan, Mongolia and China. Hence, the epizootic situation on these diseases should be constantly monitored, controlled, adapted and extrapolated to specific periods of warning [14].

The trend for aggravation of SP and GP epizootic situation intensity had been dynamically predicted up to 2020 in potentially infected regions, however, taking into account cyclical fluctuations of the situation, it was assumed that the intensity level might reduce in 2017–2020. The maximum registration frequency of these nosounits was characteristic of regions with a higher probability of SP and GP emergence [1].

During the study period (2000–2021: 22 years) 114 outbreaks of SP (65) and GP (49) were detected in the RT: 14 – in the Sughd Region (only SP), 70 – in the Khatlon Region (SP – 44, GP – 26) and 30 – in the RRS (SP – 7, GP – 23) (Table 2). The largest number of outbreaks (35) in the Republic was registered in 2002 (SP – 7, GP – 28): in the Sughd Region, the same parameter was 4 (2008: SP), in the Khatlon Region – 27 (2002: SP – 7, GP – 20) and in the RRS – 8 (2002: GP) (Fig. 1).

While only SP outbreaks were identified in the Sughd Region, the outbreak number for SP exceeded the corresponding indicator for GP in the Khatlon Region and GP was much more frequent than SP in the RRS. Most SP outbreaks were detected in the Khatlon Region (67.69%), followed by the Sughd Region (21.54%) and the RRS (10.77%), and the corresponding indicators for GP in the Khatlon Region and the RRS were 53.06 and 46.94%, respectively. The distribution of both disease outbreaks was as follows: the Sughd Region (SP only) – 12.28%, the Khatlon Region – 61.4%, the RRS – 26.32%. In total 57.02% of outbreaks were associated with SP and 42.98% – with GP in Tajikistan (Fig. 2).

Sheep pox in the Sughd Region was registered for 9 years (SR 0.41 – average; MNODRP – 1.56; MNOOP – 0.64), in the Khatlon Region – 13 years (SR 0.59 – average; MNODRP – 3.38; MNOOP – 2) and in the RRS – 5 years (SR 0.23 – below average; MNODRP – 1.4; MNOOP – 0.23), and GP in the Khatlon Region was registered during 5 years (SR 0.23 – below average; MNODRP – 5.2; MNOOP – 1.18) and in the RRS – 9 years (SR 0.41 – average; MNODRP – 2.56; MNOOP – 1.05) (Fig. 3). The infection period with regard to these diseases in the RT from 2000 to 2021 was 14 years, respectively (SR 0.64 – above average; MNODRP – 4.64; MNOOP – 2.95) and 12 years (SR 0.55 – average; MNODRP – 4.08; MNOOP – 2.23), for both SP and GP – 16 years (SR 0.73 – above average; MNODRP – 7.13; MNOOP – 5.18). During 22 years of observation in the Khatlon Region the both diseases were registered for 14 years (SR 0.64 – above average; MNODRP – 5; MNOOP – 3.18) and 11 years in the RRS (SR 0.5 – average; MNODRP – 2.73; MNOOP – 1.36).

During the study period the peaks of SP incidence in the RT (Fig. 4) were registered in 2002 (7 outbreaks in the Khatlon Region), 2008 (10 outbreaks: 4 in the Sughd and 6 in the Khatlon Regions) and 2013 (5 outbreaks: 1 for each of the Sughd and Khatlon Regions, 3 in the RRS). However, the peaks of the disease outbreak detection in the regions do not always comply with the indicators for the Republic: in the Sughd Region these peaks were reported in 2004 (2 outbreaks) and 2008 (4 outbreaks – compliant data), in the Khatlon Region – in 2002 (7 outbreaks – compliant) and 2007 (8 outbreaks), in RRS – in 2013 (3 outbreaks – data compliant). Graphically, the morbidity periods are presented in ascending-descending or undulating lines: the Sughd Region – 2003–2006, the Khatlon Region – 2000–2004 and 2005–2009, the RRS – 2012–2014) and descending (the Sughd Region – 2008–2009) lines. In general, 3 waves of SP morbidity were identified in Tajikistan: 2000–2005, 2006–2009, and 2012–2014. One outbreak was identified in 2018 in the Khatlon Region.

To be described among the last ones [15] were the pox outbreaks (caused by the SP virus according to NCFSD data) that occurred in February 2018 in the Dangara Raion of the Khatlon Region in two mixed flocks among 1.5–2 month old lambs at an early lambing stage, that had been infected in winter pastures, and the source could not be identified (it was assumed that it could be the pathogen-infected sheep pen). The severity of clinical and pathomorphological changes was due to both the virulence of the circulating strain and the age of diseased animals (50% mortality), who lacked colostral immunity, and the animals' resistance level was low as a result of colostrum and milk underfeeding. Although these outbreaks occurred in flocks belonging to one holding at a distance of 10–12 km, however, no association was established between them.

In 2002 (28 outbreaks – undulating period), 2007 (3 outbreaks – ascending period), 2013 (4 outbreaks – undulating) and 2019 (2 outbreaks – ascending period) the GP incidence peaks were recorded in Tajikistan (Fig. 4), including the indicators for the Khatlon Region (peaks: in 2002 – 20; 2013 and 2019 – 2 outbreaks each) and the RRS (peaks: in 2002 – 8; 2007 – 3 and 2013 – 2 outbreaks). The undulating period of GP incidence in 2001–2004 was due to the situation in the RRS, and its peak was an outbreak in the Khatlon Region, where 2 GP outbreaks were also observed in 2019.

In 2000 several pox outbreaks were identified in the areas bordering on Afghanistan in the Khatlon Region of the RT [10], mainly among Angora goats with the morbidity and mortality rate of 90 and 26%, respectively. Typical lesions on a significant part of hairless skin areas were observed in animals. The disease which was most severe before and during lambing, caused anorexia, vision loss, udder lesions, decreased milk production, mass abortions and infertility, and the mortality rate among newborn goatlings was 85–90%. The following year (2001) GP was identified in 11 farms already. In 2002, mainly in the autumn-winter period, it spread in two regions of Tajikistan (the Khatlon Region and the RRS) among goats of local breeds, the disease had a mild form (mortality up to 3%) and the monopathogenic agent was not transmitted to sheep kept together.

When the pathogen was isolated in VNIIVViM (Pokrov, Russia) using electron microscopy, brick-shaped virions (180–320 nm) with rounded edges were detected in the pathological material, the virus antigen titer during solid-phase enzyme immunoassay was 1:32–1:64 [16–18]. Most capripoxvirus strains are species-specific, but some of them can cause disease in both sheep and goats. The GP

virus strain Dangarinsky isolated in the RT in 2002 was pathogenic for both goats and sheep, and the former died after they developed a full complex of disease symptoms, and the latter had a mild form of the disease (with no manifestation of characteristic clinical signs), indicating the possible circulation of the virus among non-immune sheep which could be the pathogen source in the inter-epizootic period [10, 16, 19]. However, despite the high degree of antigenic relation, SP and GP viruses are phylogenetically diverse, and the latter, often inducing a more severe form of the disease, causes high mortality in young animals and significant economic losses.

Table 2
Epizootic situation on sheep pox and goat pox in the Republic of Tajikistan (2000–2021)

Year			Region, area				RRS			Tajikistan		
			Sughd	Khatlon								
			SP	SP	GP	SP + GP	SP	GP	SP + GP	SP	GP	SP + GP
2000				2		2				2		2
2001			1	5		5		2	2	6	2	8
2002				7	20	27		8	8	7	28	35
2003			1	5		5		3	3	6	3	9
2004			2	1		1		2	2	3	2	5
2005			1	1		1				2		2
2006			1	3		3		1	1	4	1	5
2007				8		8	1	3	4	9	3	12
2008			4	6		6				10		10
2009			2	3		3	1		1	6		6
2010								1	1		1	1
2011												
2012							1	1	2	1	1	2
2013			1	1	2	3	3	2	5	5	4	9
2014			1	1	1	2	1		1	3	1	4
2015												
2016												
2017												
2018				1	1	2				1	1	2
2019					2	2					2	2
2020												
2021												
Total	outbreaks		14	44	26	70	7	23	30	65	49	114
	years	observation	22	22	22	22	22	22	22	22	22	22
		disease registration	9	13	5	14	5	9	11	14	12	16
SR			0.41	0.59	0.23	0.64	0.23	0.41	0.50	0.64	0.55	0.73
Average annual number of outbreaks per period		disease registration	1.56	3.38	5.20	5.00	1.40	2.56	2.73	4.64	4.08	7.13
		observation	0.64	2.00	1.18	3.18	0.32	1.05	1.36	2.95	2.23	5.18

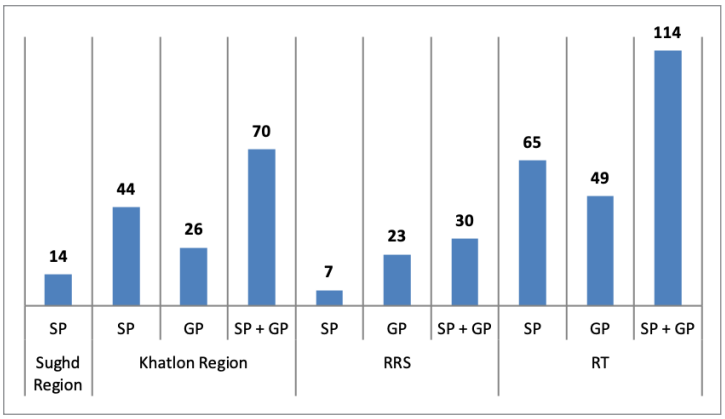


Fig. 1. Number of sheep pox and goat pox outbreaks reported in the Republic of Tajikistan in 2000–2021

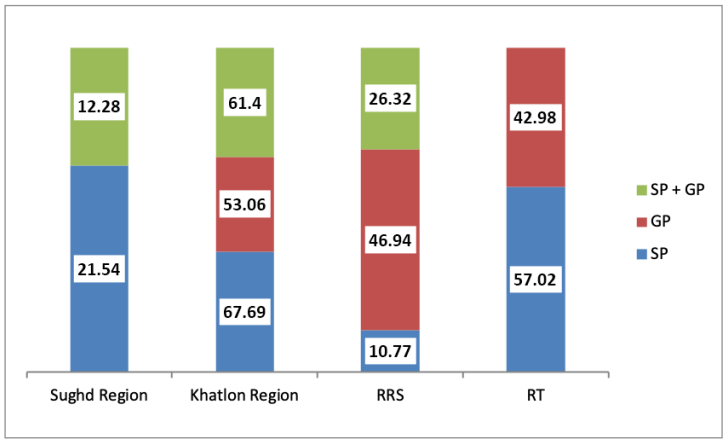


Fig. 2. Proportion of sheep pox and goat pox outbreaks in the Republic of Tajikistan in 2000–2021 (%)

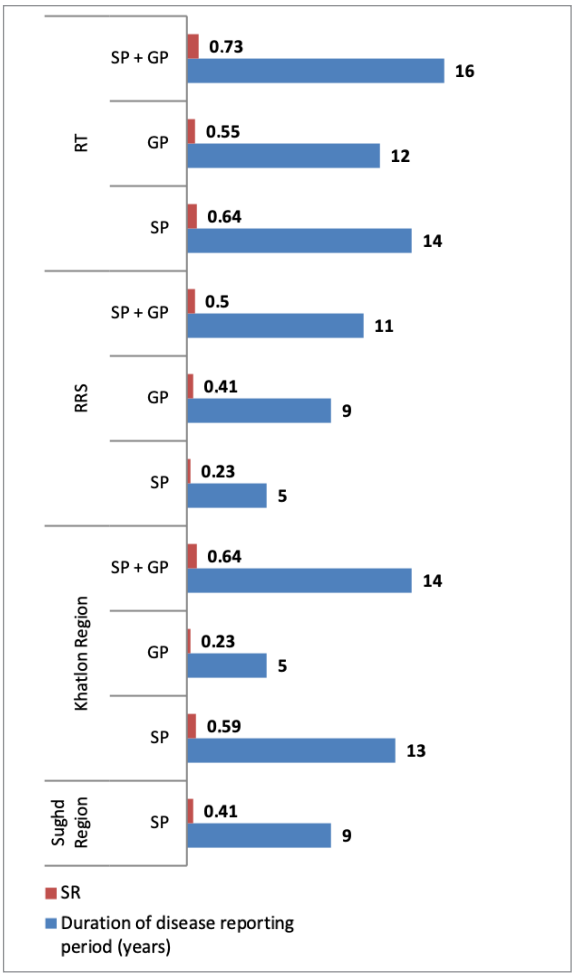


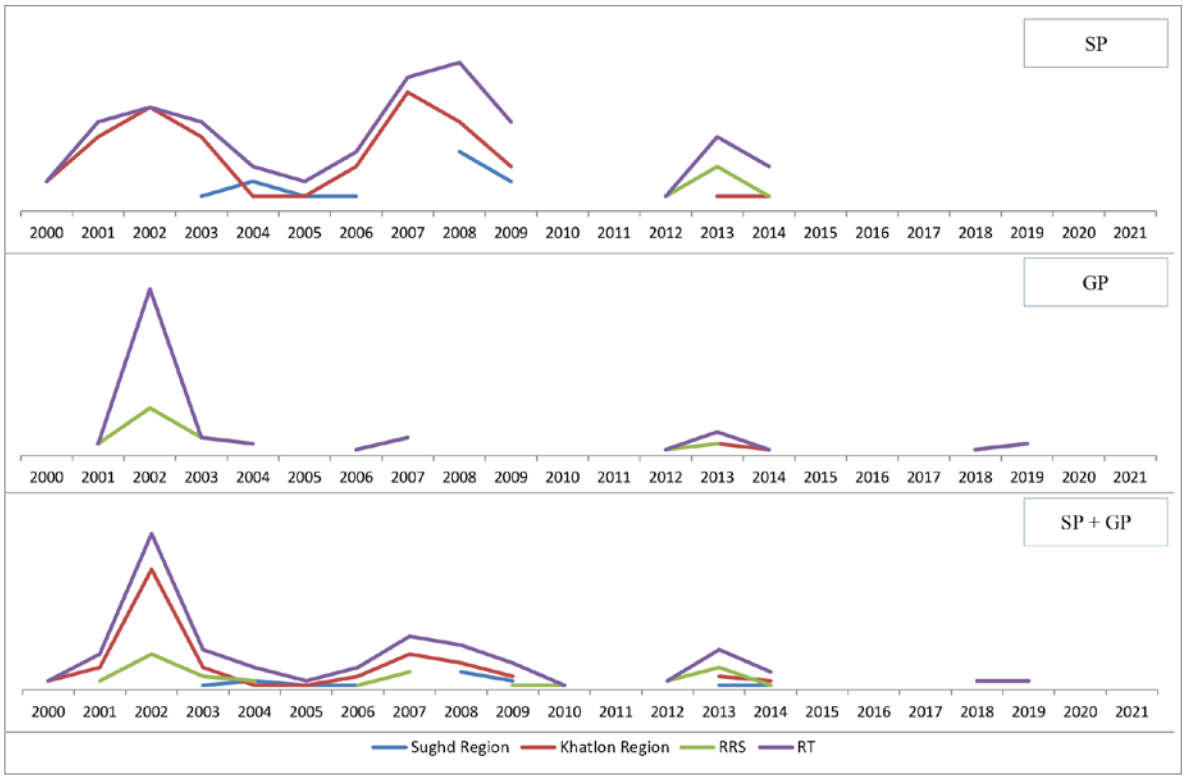
Fig. 3. Reporting period duration (years) and stationary rate for sheep pox and goat pox in the Republic of Tajikistan in 2000–2021

The specific sera obtained using SP and GP virulent viruses neutralized the SP virus strain B/5-96 and GP virus strain Dangarinsky adapted to the continuous sheep kidney cell culture in 1:32 and 1:64 titers, respectively [17, 20].

In total, the SP and GP outbreaks caused 3 waves (periods) of the disease incidence among small ruminants in Tajikistan (Fig. 4): 2000–2005 (peak in 2002 – 35 outbreaks), 2006–2010 (peak in 2007 – 12 outbreaks) and 2012–2014 (peak in 2013 – 9 outbreaks). One SP and one GP outbreaks were registered in 2018, 2 GP outbreaks – in 2019. In the Khatlon Region, 2 waves (periods) of morbidity were observed in 2000–2005 (peak in 2002 – 27 outbreaks), 2006–2009 (peak in 2007 – 8 outbreaks) and a descending period from 2013 (3 outbreaks) to 2014 (2 outbreaks). The incidence in the RRS was graphically expressed in 2 waves (periods) in 2001–2004 with a peak in 2002 (8 outbreaks) and in 2012–2014 with a peak in 2013 (5 outbreaks), as well as one ascending period in 2006–2007 (1 and 4 outbreaks, respectively). In addition, one SP and one GP outbreak were identified in this region in 2009 and 2010.

The analysis of the dynamics and structure of small ruminant pox nosoareal in the RT in 1991–2011 showed increased intensity of the epizootic situation, which was characterized by increased outbreak incidence, but the morbidity rate had a tendency to decrease. The temporal cycles of epizootic process intensity determined for Tajikistan were 6–8 and 14–16 years. When dynamic models were extrapolated to the period up to 2015, the incidence rate of outbreaks ranged from 0 to 10 per 1 million animals, the incidence coefficient was 1–10 cases per 100 thousand small ruminants ($P > 0.75$). Environmental and socio-economic factors significantly influenced the territorial distribution of intensity indicators of epizootic process: SR – 0.437, IR – 0.478 and the morbidity rate – 0.45 ($\alpha < 0.005 – 0.01$). Probability of SP and GP emergence on the RT territory for the Khatlon Region, south and southwest of the Gorno-Badakhshan Autonomous Region was 0.6–0.8 (above average), for the Sughd Region and the RRS – 0.4–0.6 (average) [13].

Analysis of the SP epizootic process dynamics in Tajikistan in 2000–2021 revealed that in 2000–2009 it was mainly determined by the situation in the Khatlon Region, while the situation in the Sughd Region had similar trends in 2003–2005, 2008 and 2009, and in the RRS – in 2012–2014. The development of the GP epizootic process in the RRS in 2001–2004, 2006, 2007, 2012, 2013 formed the corresponding situation in the RT, which was determined by the situation in the Khatlon Region in 2013, 2014, 2018



Puc. 4. Dynamics of sheep pox and goat pox epizootic process in the Republic of Tajikistan in 2000–2021 (outbreaks)

and 2019. It is unquestionable that the SP and GP epizootic process in Tajikistan depends on the situation in the Khatlon Region, but as regards SP, it is significantly affected by the situation in the Sughd Region , and as regards GP – by the situation in the RRS.

The SP and GP tensity decreased in 2017–2020 due to cyclical fluctuations of the epizootic situation: no outbreaks were registered in 2017, and in 2018 (SP – 1, GP – 1) and 2019 (GP) there were 2 outbreaks in the Khatlon Region, which confirms trueness of the previously developed model of potential nosoareal development [1].

Average annual number of SP and GP outbreaks within the disease reporting period in the RT (Fig. 5) was 7.13 (SP – 4.64; GP – 4.08). This indicator was the highest in the Khatlon Region (5.00: SP – 3.38; GP – 5.2), in descending order followed by the RRS (2.73: SP – 1.40; GP – 2.56) and the Sughd Region (SP – 1.56). It is characteristic that in the regions of GP distribution this indicator significantly exceeded the corresponding one (in the Khatlon Region by 1.5; in the RRS – by 1.8 times) for SP. Average annual number of SP and GP outbreaks within the observation period can be considered as preliminary prognostic:

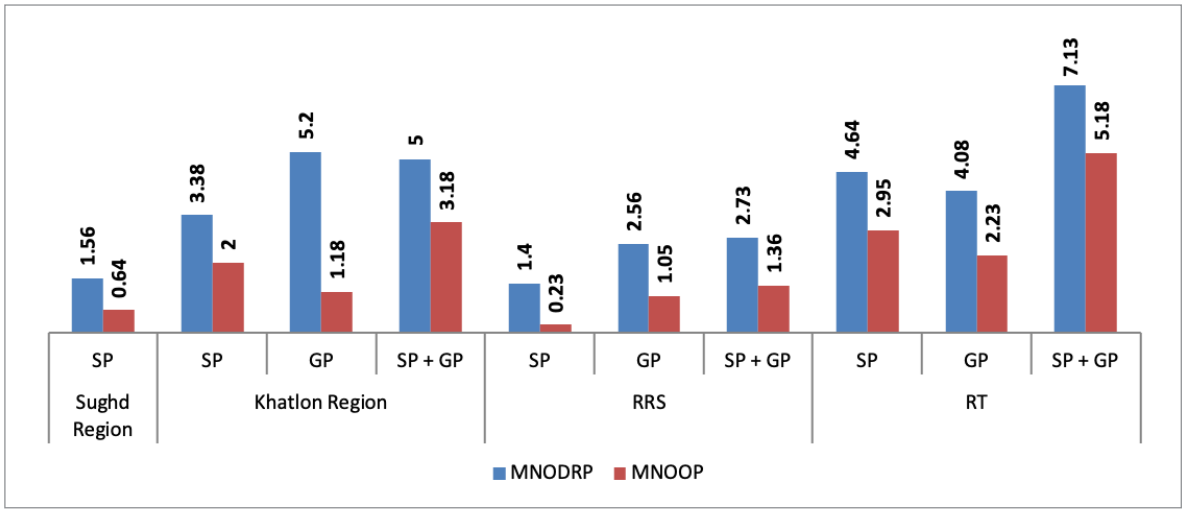


Fig. 5. Average number of sheep pox and goat pox outbreaks (annually) in the reporting and observation periods (2000–2021) in the Republic of Tajikistan

in general in Tajikistan – 5.18 (SP – 2.95; GP – 2.23), in the regions – from 0.64 (SP), in the Sughd Region to 3.18 (SP – 2.00; GP – 1.18) in the Khatlon Region, the corresponding values for the RRS – 1.36 (SP – 0.23; GP – 1.05).

Analysis of the SP and GP epizootic situation dynamics in Tajikistan in 2000–2021 identified the main causes of outbreaks of these diseases: 1) vaccination coverage of not all livestock population; 2) immunization without taking into account the colostral immunity; 3) contact of healthy animals with diseased ones when being driven to pastures (winter and summer) and grazing on high-altitude (summer) pastures; 4) grazing of small ruminants on permanently infected pastures bordering on Afghanistan; 5) vaccination of emaciated animals; 6) non-compliance with veterinary and sanitary and quarantine rules.

CONCLUSION

Characteristics and patterns of SP and GP epizootic process spread and manifestations identified based on systemic epizootological analysis of the structure and dynamics of nosoareals, as well as the results of the risk assessment of introduction, emergence and spread (taking into account climatic, socio-economic and organizational and managemental factors), monitoring and identification of SP and GP infected and endemic zones confirm the need for a systematic approach to epizootological surveillance of highly dangerous and economically significant infectious diseases that have a trend for transboundary spread. This approach will facilitate solving the issue of possible hazard analysis and implementation of veterinary and sanitary measures in case of disease emergence in any country to ensure epizootological well-being.

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Testing of chickens experimentally infected with A/H9N2 avian influenza virus isolates for their immune responses

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SUMMARY
Data on tests of chickens for their immune responses to infection with low pathogenic A/H9N2 avian influenza virus isolates belonging to Y-280 and G1 genetic lines are presented in the paper. $CD4^+/CD8^+$ ratios were determined with flow cytometry for initial immune status examination and for detection of apparent immune system disorders. Quantitative analysis of peripheral blood lymphocyte subpopulations in chickens revealed changes characteristic of the immune suppression. Analysis of dynamics of T- and B-lymphocyte levels in blood of the infected chickens revealed decrease in relative T-lymphocyte counts and increase in relative B-lymphocyte counts. T-lymphocyte subpopulation composition expressed as $CD4^+/CD8^+$ ratio (%) changed after the infection: $CD4^+$ cell proportion was found to decrease whereas $CD8^+$ cell proportion increased. According to literature data, immune response activated by vaccination induces the reverse dynamics towards to increase in $CD4^+/CD8^+$ ratio. Both cell-mediated immunity and humoral immunity play role in development of the immune response in chickens infected with avian influenza viruses. Apparent humoral immune response was detected by serological tests of sera taken from chickens on day 14 after infection. Mean specific anti-A/H9N2 AIV antibody titre in all groups of test chickens infected with low pathogenic avian influenza virus isolates was higher than 6 $\log_2$. High level of specific antibodies to avian influenza virus was indicative of postvaccinal humoral immune response development.

Keywords: avian influenza virus (AIV), H9N2, T-cells

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Изучение иммунного ответа цыплят после экспериментального заражения изолятами вируса гриппа птиц A/H9N2

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РЕЗЮМЕ

Представлены данные по изучению параметров иммунного ответа цыплят после инфицирования изолятами низкопатогенного вируса гриппа птиц подтипа A/H9N2, относящимися к генетическим линиям Y-280 и G1. Для первичного исследования иммунного статуса и выявления выраженных нарушений иммунной системы были определены соотношения $CD4^+/CD8^+$ клеток методом проточной цитофлуориметрии. В результате количественного анализа субпопуляций лимфоцитов периферической крови цыплят обнаружено наличие изменений, характерных для иммунной супрессии. При изучении динамики уровня Т- и В-лимфоцитов в крови инфицированных цыплят установлено снижение относительного количества Т-лимфоцитов и увеличение относительного количества В-лимфоцитов в крови. После инфицирования изменение субпопуляционного состава Т-лимфоцитов в процентном соотношении $CD4^+/CD8^+$ клеток отмечено в сторону уменьшения процента $CD4^+$ клеток и увеличения процента $CD8^+$ клеток. Согласно литературным данным, при иммунизации вакцинными препаратами активация иммунного ответа приводит к обратной динамике в сторону увеличения отношения $CD4^+/CD8^+$ клеток. В формировании иммунного ответа у цыплят после инфицирования вирусами гриппа птиц играет роль не только клеточно-опосредованный, но и гуморальный иммунитет. В результате серологических исследований сывороток крови цыплят после инфицирования на 14-е сут установлен выраженный гуморальный иммунный ответ. Средний титр специфических антител к вирусу гриппа птиц подтипа A/H9N2 во всех группах цыплят, зараженных изолятами низкопатогенного вируса гриппа птиц, был выше 6 $\log_2$. Высокий уровень специфических антител к вирусу гриппа птиц показал развитие постинфекционного гуморального иммунного ответа.

Ключевые слова: вирус гриппа птиц, H9N2, Т-клетки

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INTRODUCTION

Low pathogenic A/H9N2 avian influenza virus is a RNA virus belonging to *Orthomyxoviridae* family, *Alphainfluenzavirus* genus, *Influenza* A virus species [1]. The virus was reported for the first time in birds in Wisconsin State, USA, in 1966 [2, 3]. Since then, the low-pathogenic A/H9N2 avian influenza virus has become widespread worldwide, especially in Asia and the Middle East [4–6]. In mainland China, A/H9N2 influenza virus was first isolated in 1994 and has become the most common subtype of avian influenza virus in poultry [4, 7].

This virus is responsible for development of clinically pronounced disease in case of concurrent infection with opportunistic viral and bacterial pathogens and poses a constant threat to poultry industry [8–11]. Economic losses are resulted from the following: increased mortality in young birds, decrease in egg and meat production in poultry establishments.

Preventive immunization against A/H9N2 avian influenza aimed at reducing economic losses is used by many countries (China, Pakistan, Iran, Israel, South Korea, etc.) for this disease control [2, 12–15]. In the Russian Federation, programmes for health status improvement and infection eradication can include preventive immunization with inactivated vaccines due to A/H9N2 AI virus circulation [16].

Vaccination induces both humoral and cell immunity. Cell mechanisms play the major role in immune response to viruses. T-lymphocytes are the main cells of acquired anti-virus immunity. Among them, $CD8^+$ T-lymphocytes recognize foreign viral antigens associated with class I histocompatibility molecules and kill cells infected with viruses. $CD4^+$ T-lymphocytes (T-helper cells) recognize viral antigens located on antigen-presenting cells associated with class II histocompatibility molecules and act as assistants in the synthesis of specific antiviral antibodies by B-lymphocytes [17]. Antigen-recognizing $CD8^+$ T-lymphocytes play a critical role in specific cell-mediated response [18, 19]. Increase in relative $CD8^+$ T-cell count was observed in chickens infected with A/H7N9 and A/H9N2 AI viruses and these cells were demonstrated to confer antivirus protection [20, 21]. However, according to literature data, immunization of chickens against A/H9N2 AI virus was found to result in increase in relative $CD4^+$ T-cell count and decrease in $CD8^+$ T-cell count [21, 22]. $CD4^+/CD8^+$ ratio of T-cells are determined for initial immune status examination and for apparent immune system disorder detection. According to the published data, $CD4^+/CD8^+$ ratio markedly increased after immunization and apparently decreased after infection that supposed immunity enhancement after immunization and immunity suppression after infection [23–26]. $CD8^+$ T-cell deficiency could be a reason for insufficient anti-virus immune response after poultry vaccination and infection of poultry in vaccinated flocks in China.

It is also important to study humoral response since low pathogenic AI virus is able to induce immune suppression in case of co-infection with other pathogens. Various infectious diseases become more severe when the immunity is suppressed.

Thus, a comprehensive study of the immune response features in chickens experimentally infected with low-pathogenic A/H9N2 avian influenza virus is of considerable interest.

MATERIALS AND METHODS

Virus. Low pathogenic A/H9N2 avian influenza virus isolates belonging to Y-280 genetic line (A/chicken/Tadjikistan/2379/2018, A/chicken/Primorsk/419/2018) and G1 genetic line (A/chicken/Chelyabinsk/30/2019) recovered and identified in the FGBI "ARRIAH" Reference Laboratory for Avian Viral Diseases were used for infection during the experiment.

Virus isolation was carried out in 10 day-old specific pathogen free (SPF) chicken embryonated eggs. 10–20% suspension was prepared from the biological material with phosphate buffered solution (pH 7.2–7.4) and injected into allantoic cavity of chicken embryonated eggs, 0.2 cm^3 per egg. Extraembryonic fluid was collected from the chicken embryonated eggs with embryos dead 24 or more hours of incubation for further examinations. Extraembryonic fluid with infectivity of 10^6 EID_{50}/cm^3 and hemagglutination titre of 9 log_2 was used for infection of chickens.

Experiment in animals. Egg cross 30 day-old chickens without antibodies to avian influenza virus obtained from infectious disease-free holdings were used for the experiment. Chickens were divided into three groups, 5 chickens per group, and kept in isolators. Virus-containing extraembryonic fluid with infectivity of 10^6 EID_{50}/cm^3 was injected to chickens intramuscularly in a volume of 0.5 cm^3. Blood samples were collected from chickens before infection and for 14 days after infection for serological tests for

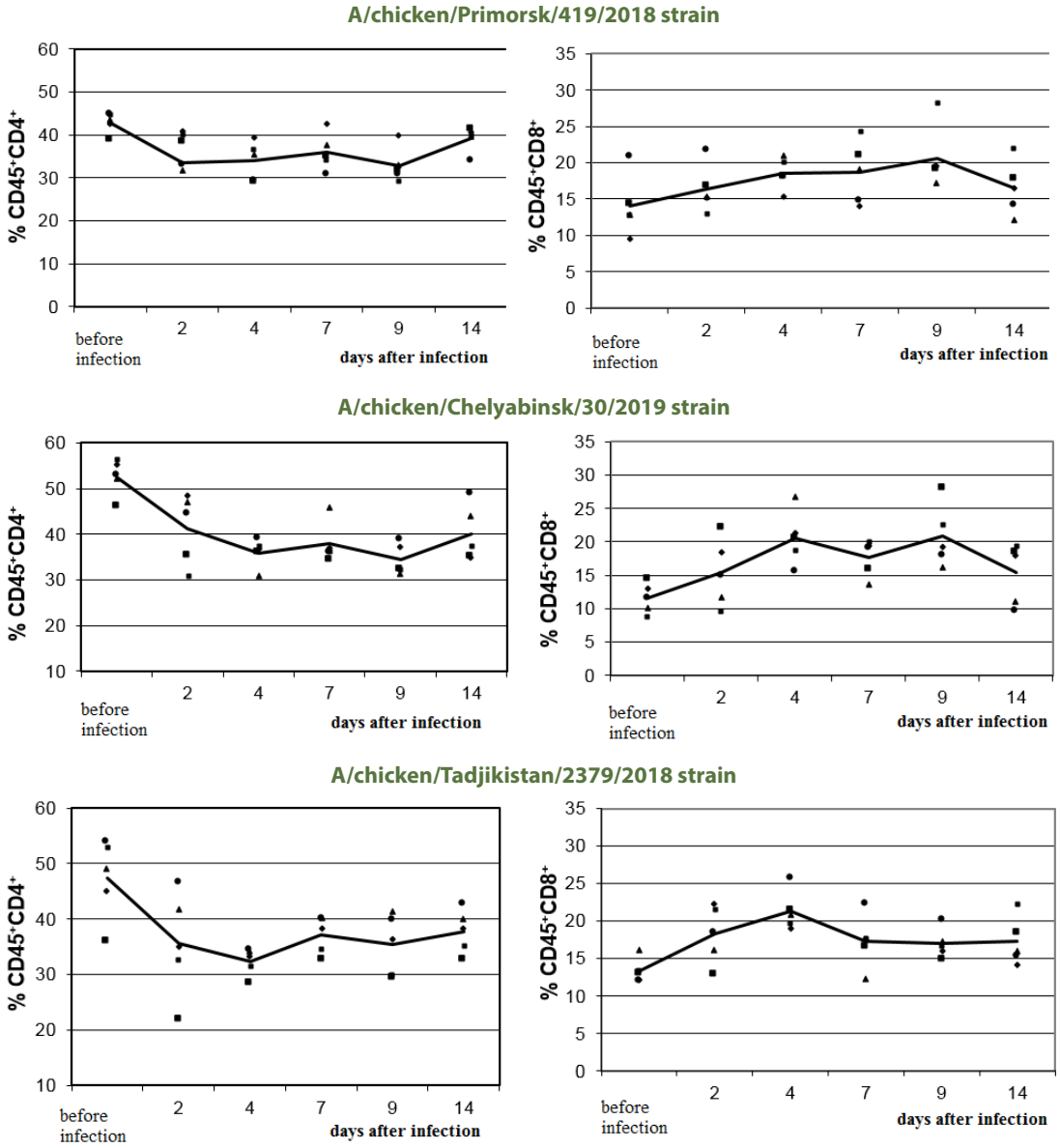

Fig. 1. Dynamics of T-cell subpopulations in chickens after their infection with H9N2 avian influenza virus isolates.
Solid line – arithmetic mean for group of 5 chickens; individual symbols – the percentage of cells for each chicken in the group

immune response with hemagglutination inhibition test and with flow cytometry.

All experiments in animals were carried out in strict accordance with the interstate standards on laboratory animal keeping adopted by the Interstate Council for Standardization, Metrology and Certification as well as according to Directive 2010/63/EU of European Parliament and the Council of 22 September 2010 about protection of animals used for scientific purposes.

Serological tests. Sera collected before and 14 days after infection were tested for antibodies to A/H9N2 avian influenza virus with hemagglutination inhibition (HI) test using commercial test-kit produced by the FGBI "ARRIAH" (Vladimir) in accordance with the instruction for its use. Before testing, sera were inactivated by heating at 56 °C for 30 minutes. Test results were recorded visually after complete red blood cell sedimentation in control wells (in the form of "button"). Test results were considered positive when the test sera contained specific antibodies to A/H9N2 avian influenza virus at a titre of 1:16 (4.0 $\log_2$) or higher.

Quantitative analysis of lymphocyte subpopulations. Dynamics of changes in the proportions of T-lymphocyte ($CD45^+CD3^+$, $CD45^+CD4^+$ and $CD45^+CD8^+$) and B-lymphocyte ($CD45^+$, $CDBu1a^+$) populations in the peripheral blood of chickens was examined by flow cytometry. For this purpose, blood samples were collected from chickens before and on day 2, 4, 7, 9 and 14 after infection in tubes containing K3-EDTA anticoagulant.

Lymphocytes were isolated from chicken peripheral blood according to standard method [27] using Ficoll-Paque™ PLUS medium for lymphocyte separation (BioWest, France). Labeled monoclonal antibodies, CD45-FITC, CD4-PE, CD8α-PE, CD3-PE and Bu1a-PE (Southern Biotech, USA), were used for sample preparation for lymphocyte surface marker detection. Lymphocyte samples (50 µl) were added to microtubes in several replicates (depending on number of used antibody panels). Fluorochrome-conjugated monoclonal antibodies (2 µl) were added and the tubes were incubated for 30 minutes at temperature of 4–8 °C. Unbound monoclonal antibodies were removed by centrifugation with phosphate buffered solution at 260 g for 10 minutes. BD FACS Calibur flow cytometer (Becton Dickinson, USA) was used for quantitative analysis of cells. Cell Quest Pro 1.0 software was used for obtained result assessment and processing.

Statistical analysis of the results. Statistica 10.0 programme was used for data statistical processing.

RESULTS AND DISCUSSION

Cell and humoral immunities were assessed in chickens after their infection with different three H9N2 avian influenza virus isolates.

The following clinical signs were observed in chickens on day 4 and day 7 after infection: depression, ruffled feathers, refusal from feed. No deaths were recorded in the infected chickens.

Blood samples collected from chickens before and on day 2, 4, 7, 9 and 14 after infection were examined with flow cytometry for quantitative analysis of lymphocyte subpopulations.

Significant decrease in $CD45^+CD4^+$ T-cell (T-helper) proportion and increase in $CD45^+CD8\alpha^+$ cytotoxic cell proportion were reported in chicken blood 2–4 days after their infection (Fig. 1). Relative counts of both populations in infected chickens differed from the initial levels (before infection) by 1.3–1.5 times for T-helpers and by 1.3–1.9 times for cytotoxic cells.

Increase in T-helper level in the blood was observed 9 days after infection. Both T-lymphocyte populations in infected chickens returned to normal levels by day 14 after infection but not in all tested chickens.

Progress of the infection had a suppressive effect on the immune system of the infected chickens. Obtained data on decrease of relative T-helper concentration in peripheral blood after infection confirm the data obtained by X. Hao et al. [21] and M. Dai et al. [22]. Significant increase

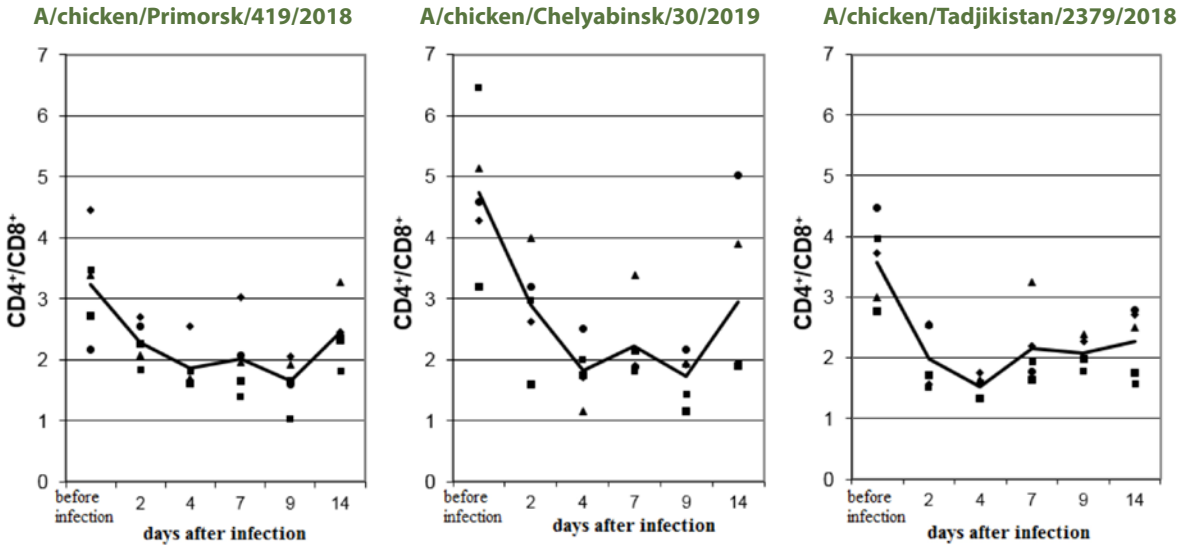

Fig. 2. Dynamics of $CD4^+/CD8^+$ ratio in chicken blood lymphocytes after infection with three H9N2 avian influenza virus isolates.
Solid line – arithmetic mean for group of 5 chickens;
individual symbols – the percentage of cells for each chicken in the group

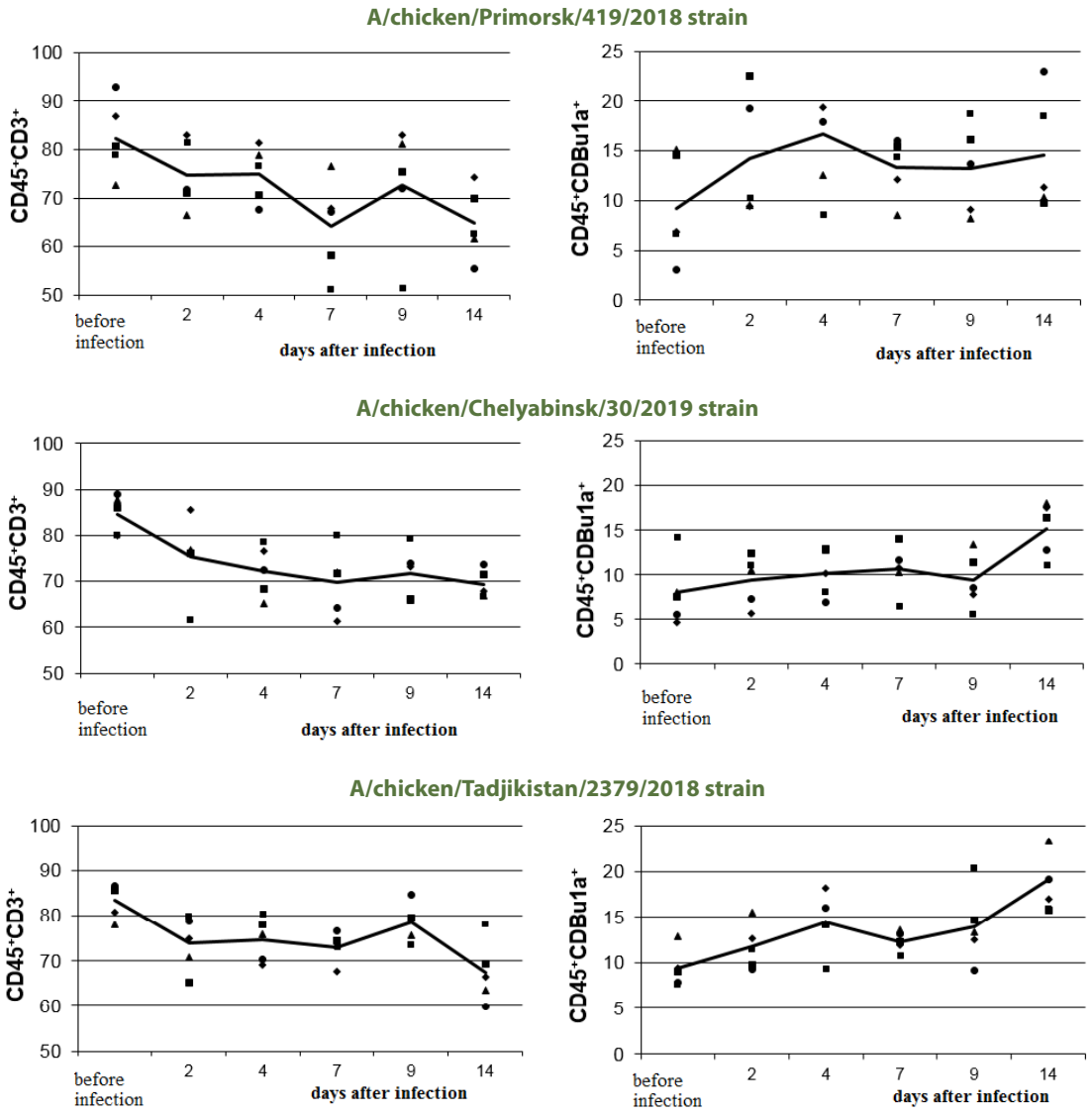

Fig. 3. Dynamics of T- and B-lymphocyte levels in chicken peripheral blood after infection with three H9N2 avian influenza virus isolates.
Solid line – arithmetic mean for group of 5 chickens;
individual symbols – the percentage of cells for each chicken in the group

in cytotoxic ($CD8^+$) T-cell proportion in blood of chickens infected with A/H9N2 avian influenza virus on day 5–7 after infection was also reported by M. Dai et al. [22].

Analysis of changes in $CD4^+/CD8^+$ ratio showed that it decreased by 1.9; 2.6 and 2.3 times in chickens of group 1, 2 and 3, respectively, on day 4 after infection due to decrease in relative $CD4^+$ counts and increase in a $CD8\alpha^+$ T-lymphocytes (Fig. 2). $CD4^+/CD8^+$ ratio increased again by day 14 after infection but it remained averagely 1.3–1.6 times lower than the initial one.

Yang Y. et al. [28] and Dai M. et al. [22] also demonstrated that viral infections in chickens induced immune suppression manifested, among others, by decrease in $CD4^+/CD8^+$ ratio in blood T-lymphocytes. On the contrary, vaccination gave rise to immune response activation and reverse dynamics towards to increase in $CD4^+/CD8^+$ ratio [15, 22].

Xue M. et al. [23] and Yang S. et al. [24] believed that an increase in the $CD4^+/CD8^+$ ratio after immunization and a decrease in the $CD4^+/CD8^+$ ratio after infection suggested that immune response enhanced after vaccination and immunity was suppressed in case of viral infection. Vaccination induced a pronounced humoral immune response and $CD4^+$ T-cell-mediated response.

Studies performed by L. Fu et al. [25] and M. Dai et al. [26] showed that the virus infection mainly stimulated $CD8^+$ T-cell response, and immunization stimulated $CD4^+$ T-cell response. High level of antibodies to A/H9N2 avian influenza virus and an increase in cytotoxic $CD8^+$ T-cell proportion play an important role in anti-virus protection [29, 30].

Figure 3 shows dynamics of relative T- and B-lymphocyte counts in infected chicken blood. Decrease in T-lymphocyte counts averagely by 15–20% was reported in all three groups that was indicative of insufficient cell-mediated immunity. Increase in relative B-lymphocyte counts by 5–10% depending on the group was reported as early as by day 14 after infection. Together with an increase in

the T-helper proportion, this demonstrated the activation of the immune response in infected chickens.

The function of B-lymphocytes responsible for humoral immunity is to transform B-cells into plasma cells secreting immunoglobulins having specific activity against the invaded antigen. Assessment of the dynamics of the relative B-lymphocyte counts in the blood of infected chickens revealed that they increased.

Chicken sera were HI tested before and on day 14 after infection. Results of tests for specific antibodies to A/H9N2 avian influenza virus are given in the Table.

Mean HI antibody titre in chickens of all groups was higher than 6 $\log_2$ on day 14 after infection. High anti-AIV antibody level was indicative of pronounced post-infection humoral immune response development.

Dai M. et al. [22] during comparative analysis of the key factors of immune protection of chickens infected with the A/H9N2 virus and SPF chickens immunized with an inactivated vaccine concluded that the lack of $CD8^+$ T-cells was a key cause of immunodeficiency and infection of poultry in vaccinated flocks.

Table
Results of HI tests of chicken sera for specific antibodies to A/H9N2 avian influenza virus

Isolate	Before infection		On day 14 after infection	
	Total number of samples/ positive samples	Mean antibody titre, $\log_2$	Total number of samples/ positive samples	Mean antibody titre, $\log_2$
A/chicken/ Tadjikistan/2379/2018	15/0	0	15/15	8.7 ± 0.3
A/chicken/ Primorsk/419/2018	15/0	0	15/15	8.1 ± 0.4
A/chicken/ Chelyabinsk/30/2019	15/0	0	15/15	6.9 ± 0.4

CONCLUSION

Key factors of immune response of chickens infected with various A/H9N2 avian influenza viruses were examined. Quantitative analysis of peripheral blood lymphocyte subpopulations in chickens infected with three A/H9N2 avian influenza virus isolates revealed the following changes caused by the virus infection: decrease in relative T-lymphocyte counts in blood, significant changes in T-lymphocyte subpopulation composition towards to decrease of $CD4^+$cell proportion and increase in $CD8^+$ cell proportion and, as a result, decrease in $CD4^+/CD8^+$ ratio. Assessment of the dynamics of T- and B-lymphocyte levels in blood of infected chickens showed decrease in relative T-lymphocyte counts and increase in relative B-lymphocyte counts. HI tests demonstrated pronounced humoral immune response. No significant differences both in humoral and cell immune responses were detected in chickens infected by three different low pathogenic A/H9N2 avian influenza virus isolates.

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Infectious bursal disease virus: identification of the novel genetic group and reassortant viruses

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SUMMARY

The results of the phylogenic analysis of the nucleotide sequence of the IBDV A and B genome segments have been presented. Traditionally the IBDV isolates are classified based on the phylogenic analysis of the hypervariable region of the VP2 gene. The analysis of the VP2 gene segments of the isolates detected in the Russian Federation demonstrated that most of them belong to the genetic group comprising very virulent IBDV isolates. However, not all isolates belonging to one genetic group have the same phenotypic characteristics. This is related to the fact that the virulence is determined not only based on the characteristics of the VP2 gene (A segment) but on the characteristics of the VP1 gene (B segment) as well. The IBDV genome segmentation allows formation of reassortant viruses which can be identified as a result of the genome segment analysis. The phylogenic analysis of the nucleotide sequences of VP2 and VP1 genes of 28 IBDV isolates detected at RF, Ukrainian and Kazakh poultry establishments in 2007 and 2019 showed that 15 of them are reassortant viruses. Different combinations of the genome segments have been identified among these reassortant viruses. Detection of different combinations of IBDV genome segments is indicative of the fact that the heterogeneous virus population circulates on the poultry farms. Pathogenicity studies of the three IBDV isolates showed that the most virulent was an isolate having two genome segments characteristic of the very virulent virus. Two reassortant viruses having only one genome segment A or B, characteristic of the infectious bursal disease, demonstrated less pronounced virulent properties.

Keywords: infectious bursal disease virus, phylogenic analysis

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Вирус инфекционной бурсальной болезни: выявление новой генетической группы и реассортантов

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РЕЗЮМЕ

Представлены результаты филогенетического анализа изолятов вируса инфекционной бурсальной болезни по нуклеотидным последовательностям фрагментов геномных сегментов A и B. Традиционно изоляты вируса инфекционной бурсальной болезни классифицируют на основе филогенетического анализа гипервариабельной области гена VP2. Анализ фрагмента гена VP2 изолятов, выявленных на территории Российской Федерации, показал, что большинство из них относятся к генетической группе, объединяющей высоковирулентные изоляты вируса инфекционной бурсальной болезни. Но не все изоляты, относящиеся к одной генетической группе, обладают одинаковыми фенотипическими свойствами. Это связано, в частности, и с тем, что вирулентность определяется генетическими особенностями не только гена VP2 (сегмент A), но и гена VP1 (сегмент B). Сегментированная природа генома вируса инфекционной бурсальной болезни делает возможным образование реассортантов, которые можно выявить в результате анализа обоих геномных сегментов. Филогенетический анализ нуклеотидных последовательностей фрагментов генов VP2 и VP1 28 изолятов вируса инфекционной бурсальной болезни, выявленных в птицеводческих хозяйствах РФ, Украины и Казахстана в 2007–2019 гг., показал, что 15 из них являются реассортантами. Среди реассортантов выявлены различные комбинации геномных сегментов. Выявление разнообразия комбинаций геномных сегментов вируса инфекционной бурсальной болезни свидетельствует о том, что в птицеводческих хозяйствах циркулирует гетерогенная вирусная популяция. Изучение степени патогенности трех изолятов вируса инфекционной бурсальной болезни показало, что наиболее вирулентным был изолят, имеющий оба геномных сегмента, характерных для высоковирулентного вируса. Два реассортанта, имеющих только по одному геномному сегменту A или B, характерному для высоковирулентного вируса инфекционной бурсальной болезни, обладали менее выраженными вирулентными свойствами.

Ключевые слова: вирус инфекционной бурсальной болезни, филогенетический анализ

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INTRODUCTION

The infectious bursal disease virus (IBDV) is the causing agent of the acute, highly contagious and widely spread disease in the countries with commercial poultry farming. IBDV infects lymphocytes of the bursa of Fabricius, thymus, spleen, Peyer's patches of the intestine. The consequences of IBDV-associated immunosuppression is that vaccination against other diseases is less effective and the chicks become more susceptible to opportunistic pathogens. Very virulent IBDV (VV IBDV) can cause high death rate in poultry. High contagiousness and sustainability of the virus to the environmental factors and disinfectants as well as a considerable economic losses make IBD a serous problem for poultry farming in many countries.

The infectious bursal disease virus (IBDV) is a member of the genus *Avibirnavirus*, family *Birnaviridae* [1]. These viruses have a genome that consists of two segments of double-stranded RNA designated A and B. Segment A contains partially overlapping open reading frames (ORFs). The small ORF1 encodes the non-structural VP5 virus protein, which contributes to the release of mature virions. The larger ORF2 encodes polyprotein from which VP2 (pVP2), VP4, and VP3 precursors are formed as a result of autoproteolysis [2]. VP2 protein takes part in formation of the external surface of the virion. This is the main immunogen carrying antigenic sites responsible for neutralizing the virus with antibodies. The VP2 protein is responsible for the antigenic variability and virulence of the virus. The VP3 protein interacts with all other components of the virion, so it plays a critical role in virion morphogenesis, encapsidation, and virus replication. The VP4 protein is a serine protease that plays an important role in VP2 maturation and is also involved in polyprotein proteolysis. Genomic segment B contains one ORF encoding RNA-dependent VP1 RNA polymerase responsible for viral genome replication and mRNA synthesis [3, 4].

Currently, two IBDV serotypes have been identified. IBDV strains, serotype 1, are classified into 3 types depending on the severity of the disease caused: subclinical, classical virulent and very virulent [5]. The serotype 2 virus has been isolated from turkeys and is non-virulent in chickens.

Traditionally, IBDV isolates are classified based on phylogenetic analysis of the VP2 gene segment, which includes the hypervariable region. The classification proposed by T. P. Van den Berg et al. [6], subdivides strains of serotype 1 into the following genotypes: attenuated, classical virulent, very virulent, antigenic variants, and Australian ones.

Very virulent IBDV was first detected in broilers in Europe in the late 1980s [7] and quickly spread to Africa, Asia and Latin America, causing high morbidity and mortality over 30% [8]. Antigenic variants have become predominant in the Americas. They usually cause a subclinical infection characterized by rapid atrophy of the bursa of Fabricius without significant inflammation, which can lead to immunosuppression. In 2019, a report appeared on the detection of a new IBDV antigenic variant in China

that differed from American variants and caused severe immunosuppression in chickens [9].

IBDV antigenic phenotype is determined by the VP2 hypervariable region, in particular by amino-acids located at the top of the loop and indicated as PBC, PDE, PFG, and PHI [10]. It was determined that even point mutations in these regions can cause IBDV antigenic drift [10, 11] and make vaccination against IBD ineffective.

Michel L. O. and Jackwood D. J. [12] suggested to use new IBDV classification by dividing the virus strains into 7 gene groups. Most IBDVs form three gene groups: 1 – classical, 2 – variant и 3 – VV IBDV or reassortant IBDV. The rest four genogroups include IBDVs detected in different regions of the world.

IBDV isolates belonging to one of the genogroups have several common genotypic and phenotypic properties. But not always the isolates, belonging to one and the same genogroup have the same phenotypic properties. It is related, in particular, to the fact that virulence is determined by genetic differences not only in the VP2 gene, but also in VP1 gene [13–15].

Phylogenetic analysis of the VP1 gene nucleotide sequences shows that all IBDV strains are divided into two large genetic groups: one of them contains the VV IBDV strain and the other one – all the rest IBDV strains [16].

The IBDV genome segmentation allows formation of reassortant viruses in case of host cell co-infection with different virus strains. There have been some publications on detection of reassortment between serotype 1 strains [17–22]. The pathogenicity of reassortant viruses whose A segment belongs to the VV IBDV and B segment belongs to the other non-very virulent group is lower than that of the very virulent parent strains [23]. On the other hand the reassortant virus Bpop/03 was detected in Poland which had segment A originating from VV IBDV and segment B originating from the classical attenuated strain D78 [24]. Despite the mosaicism the virus caused high mortality (80%) in experimentally infected SPF-chicks and IBD-associated lesions. These data contradict to the previous experimental studies where the natural reassortant viruses demonstrated the intermediate pathotype [23].

Analysis of the VP2 gene segment of the IBDV isolates detected in the Russian Federation in 1993–2020 demonstrated that most of them don't belong to VV IBDV group. Analysis of two genome segments are more informative and allows detection of reassortant viruses. This research describes molecular characteristics of the IBDV isolates, detected in 2007–2019 based on the alignment of neucleotide sequences of the VP2 gene hypervariable region (segment A) and VP1 gene segment containing a phylogenetic marker (segment B).

MATERIALS AND METHODS

26 IBDV isolates detected in the samples of biological material from the RF poultry farms and 2 IBDV isolates from Ukraine and Kazakhstan were used in the research (Table 1).

RNA extraction. Total RNA was extracted from the clarified suspension prepared from the bursa of Fabricius using the SV 96 Total RNA Isolation System (Promega, USA) kit.

One step *RT-PCR* was performed. To perform the test Avian Myeloblastosis Virus Reverse Transcriptase (AMV RT) and Taq-ДНК-polymerase (Promega, USA) were used according to the manufacturer's instruction. Conservative regions VP1 and VP2 were used for primer calculation. The 25 µL final dilution of the reaction mixture: 10 µL of the deionized water; 5 µL 5× buffer for RT-PCR; 2 µL of the 25 mM $MgCl_2$ solution; 1 µL of the 10 mM dNTP solution; 1 µL of the direct and reversed primer solution,10 pmol/µL concentration; 0.13 µL of the reversed transcriptase and 0.25 µL of polymerase; 5 µL of the total RNA solution. The reversed transcription was performed for 30 minutes at 50 °C. The following time-temperature parameters were

Table 1
IBDV isolates

No.	Sample receipt date	Subject where samples were collected	Isolate
1	25.06.2007	Krasnoyarsky Krai	IBDVRF02-2007
2	11.10.2007	The Republic of Dagestan	IBDVRF03-2007
3	29.10.2007	Ukraine	IBDVUkr04-2007
4	12.11.2007	Primorsky Krai	IBDVRF05-2007
5	04.06.2014	Novgorod Oblast	IBDVRF02-2014
6	28.07.2014	The Republic of Kazakhstan	IBDVKaz03-2014
7	11.08.2014	The Republic of Tatarstan	IBDVRF04-2014
8	16.10.2014	Orenburg Oblast	IBDVRF05-2014
9	08.04.2015	Vladimir Oblast	IBDVRF02-2015
10	11.10.2015	Yaroslavl Oblast	IBDVRF06-2015
11	27.10.2016	Novgorod Oblast	IBDVRF02-2016
12	09.11.2016	Chelyabinsk Oblast	IBDVRF03-2016
13	04.04.2017	Novgorod Oblast	IBDVRF01-2017
14	02.08.2017	The Chuvash Republic	IBDVRF02-2017
15	05.10.2017	Kursk Oblast	IBDVRF03-2017
16	24.11.2017	Kirov Oblast	IBDVRF06-2017
17	28.11.2017	The Mari El Republic	IBDVRF08-2017
18	21.08.2018	Leningrad Oblast	IBDVRF01-2018
19	03.08.2018	Kursk Oblast	IBDVRF02-2018
20	16.01.2019	Novgorod Oblast	IBDVRF01-2019
21	16.01.2019	Novgorod Oblast	IBDVRF02-2019
22	27.02.2019	Sverdlovsk Oblast	IBDVRF03-2019
23	07.03.2019	Leningrad Oblast	IBDVRF04-2019
24	06.05.2019	Ivanovo Oblast	IBDVRF05-2019
25	06.06.2019	Novgorod Oblast	IBDVRF06-2019
26	24.06.2019	Vladimir Oblast	IBDVRF07-2019
27	02.07.2019	Vladimir Oblast	IBDVRF09-2019
28	18.10.2019	Yaroslavl Oblast	IBDVRF10-2019

used for amplification: 95 °C – 10 min (polymerase activation), then – 40 cycles, each of them consisted of three steps: 95 °C – 50 sec., 55 °C – 50 sec., 72 °C – 60 sec. In order to increase the sensitivity the nested PCR using inner pair of primers was applied. To visualize the results of the reaction electrophoresis in 2% agarose gel with ethidium bromide was used.

Alignment of nucleotide sequences and phylogenetic analysis. Purified PCR products were used for identification of the nucleotide sequences of the VP1 and VP2 gene segments using automatic sequencer ABI Prism® 3100 (USA) and BigDye Terminator Cycle Sequencing kit (Applied Biosystems, USA) according to the manufacturer's instruction.

For analysis, we used the nucleotide sequences of IBDV isolates and strains published in the GenBank database of the NCBI electronic resource (https://www.ncbi.nlm.nih.gov/nucleotide) (Table 2). Nucleotide and corresponding amino acid sequences were analyzed using the BioEdit program, version 7.0.5.3. Sequence alignment was performed using the ClustalW multiple alignment program. The phylogenetic tree was constructed using the UPGMA algorithm in the implementation of the MEGA package, version 6.06.

BLAST program (https://blast.ncbi.nlm.nih.gov/Blast.cgi) was used for comparing nucleotide and amino-acid sequences with the database of sequences and calculation of statistical significance.

RESULTS AND DISCUSSION

The nucleotide sequences of the VP2 and VP1 gene segments were identified for 28 IBDV isolates detected on the poultry farms of the Russian Federation, Ukraine, and Kazakhstan in the period from 2007 to 2019. For comparative analysis, we used published nucleotide sequences of IBDV strains belonging to different genogroups according to the L. O. Michel and D. J. Jackwood classification [12] (Table 2).

Phylogenetic analysis of the VP2 gene segment. As a result of comparative analysis of the IBDV VP2 gene nucleotide sequences it was determined that 22 isolates belong to Genogroup 3 (VV IBDV), IBDVRF07-2019 isolate – to Genogroup 1, 2 isolates IBDVRF03-2017 and IBDVRF02-2018 are variant, 3 isolates IBDVRF05-2014, IBDVRF03-2016 and IBDVRF03-2019 differ from all isolates by 5% and form a new Genogroup 8 (Fig.).

Genogroup 3 includes VV IBDV and is the largest group. IBDV strains belonging to this group were detected during acute IBD outbreaks on poultry farms in the world. Recently the number of published nucleotide sequences of the VP2 gene of the Genogroup 3 strains has increased.

The sequence analysis shows that the hypervariable VP2 region accumulates mutations inside the group which makes it possible to identify subgroups 3-1, 3-2, 3-3 [12]. The amino-acid composition of the isolates belonging to these subgroups differ at positions 212, 222, 254, included in the loop structure and responsible for IBDV antigenic properties (Table 3).

IBDVRF07-2019 isolate, belonging to Group 1, is a derivative of the D78 vaccine strains. Nucleotide sequences of the VP1 and VP2 gene segments are 99.36% homologous to D78 strain. The attenuated vaccine based on this strain has been widely used on the RF farms for 20 years.

Two variant isolates IBDVRF03-2017 and IBDVRF02-2018 were detected in samples from one of the poultry farms of the Kursk Oblast. The VP2 gene nucleotide sequences different from each other and from the isolates of other genogroups.

Three isolates: IBDVRF05-2014, IBDVRF03-2016, IBDVRF03-2019, detected in Orenburg, Chelyabinsk and Sverdlovsk Oblasts differ from all tested and published strains and are likely to form Genogroup 8. The nucleotide sequences of VP2 gene strains and IBDV isolates, contained in the GenBank differed from the consequences of this group of isolates by more than 5%. Genogroup 8

Table 2
Nucleotide sequences of the IBDV isolates used for analysis

No.	Strain	Number in the GenBank VP2	Number in the GenBank VP1	Country	Genogroup
1	D78	AF499929	EU162090	Luxembourg	1
2	228E	AF457104	AJ878657	The Netherlands	1
3	52/70	HG974565	HG974566	Great Britain	1
4	STC	D00499	JQ619639	The USA	1
5	Variant E	AF133904	AF133905	The USA	2
6	UK661	X92760	X92761	Great Britain	3
7	624Russia	MF142552	MF142481	Russia	3-2
8	593Russia	MF142550	MF142479	Russia	3-3
9	MG4	JN982252	–	Brazil	4
10	Mexico04M101	DQ916210	–	Mexico	5
11	ITA-02	JN852986	–	Italy	6
12	002-73	AJ878908	AJ878639	Australia	7

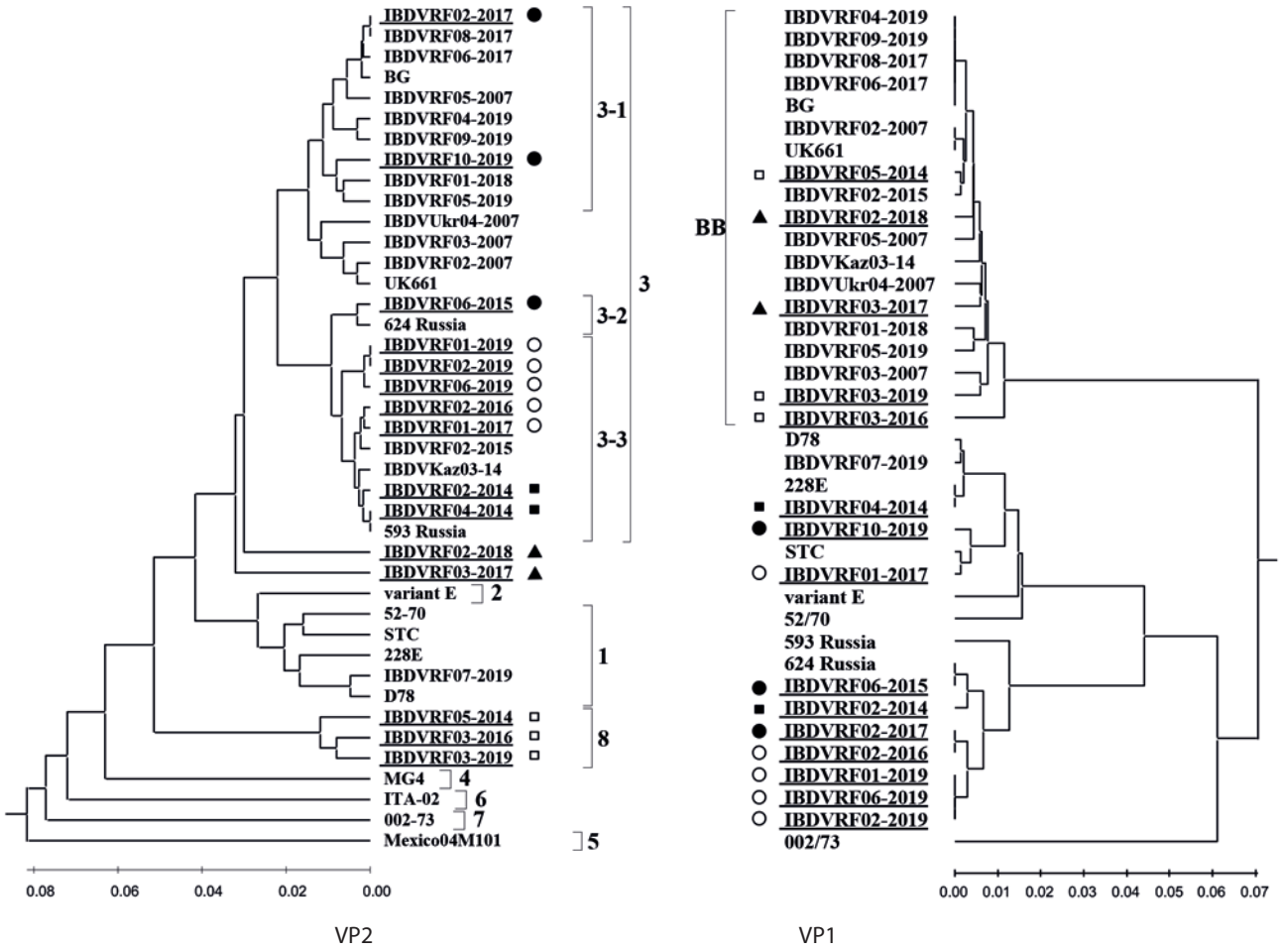

Fig. Phylogenic trees built on the basis of the alignment of the nucleotide sequences of the VP2 gene hypervariable region and VP1 gene segment.
Numerals represent genotypes, underlining and geometric figures represent reassortant isolates

isolates have VV IBDV associated amino-acid residues in the hypervariable region of the VP2 region (Table 3): 242I, 256I, 294I, 299S, as well as a number of amino acid residues peculiar only to them: 213E, 220F, 222V, 269S, 280T, 317R, 324P. It is worth noting that the amino acid change at position 222 is important because this residue is located in the PBC loop. It is believed that the transition from Pro to Thr at position 222 played a significant role in the significant change in the antigenic properties of variant IBDV strains in the 1980s [25], which led to the ineffective vaccination.

Phylogenetic analysis of the VP1 gene segment. The analysis of the published nucleotide sequences of the VP1 gene showed that IBDV strains are divided into 2 large groups. The first one includes VV IBDV, and the second includes all other genetic groups: attenuated, classical variant, antigenic and Australian variants, as well as serotype 2 [26].

It was determined that VP1 contains a TDN marker triplet characteristic of VV IBDV at positions 145/146/147 [27, 28]. Low variability of this triplet in VP1 was observed in VV IBDVs.

Phylogenetic analysis of the VP1 gene segment of the IBDV isolates under study showed that they are also divided into 2 groups: the group with the VV IBDV-like segment included 17 isolates, and the group with the non-VV IBDV-like B segment included 11 isolates. In the first group, with the exception of the isolate IBDVRF03-2017, the VP1 sequence carries the IBDV characteristic TDN marker sequence (Table 3).

Phylogenetic analysis of the VP1 gene segment shows that the group with a non-VV IBDV-like segment is divided into two subgroups. The first includes attenuated strains, classical virulent and antigenic variants, and 4 isolates under study: IBDVRF07-2019, IBDVRF04-2014, IBDVRF10-2019, IBDVRF01-2017. The second subgroup consists of 4 isolates: IBDVRF02-2014, IBDVRF02-2016, IBDVRF01-2019, IBDVRF06-2019, detected on one poultry farm in the Novgorod Oblast and three isolates: IBDVRF02-2017, IBDVRF06-2015, IBDVRF02-2019, detected in Chuvashia, Yaroslavl and Novgorod Oblasts. The same subgroup includes isolates 593 Russia and 624 Russia, detected in the RF. The origin of the IBDV isolates included in this endemic subgroup is interesting, since it includes isolates found exclusively in the territory of the Russian Federation. The comparison with the published VP1 sequences showed that the difference between IBDV nucleotide sequences of the VP1 gene and other isolates and strains was 6%. All isolates in this subgroup have NEG marker triplet.

Table 3
Difference between the amino acid sequences in the hypervariable VP2 region and VP1 marker sequence

Strain/isolate	Genogroup	VP2													VP1
		212	213	220	222	242	254	256	269	280	294	299	317	324	145-146-147
228E	1	D	D	Y	S	V	G	I	T	N	L	N	S	Q	NEG
D78	1	D	D	Y	P	V	G	V	T	N	L	N	S	Q	NEG
52-70	1	D	D	Y	P	I	G	V	T	N	L	N	S	Q	NEG
STC	1	D	D	Y	P	V	G	V	T	N	L	N	S	Q	NED
Variant E	2	D	N	Y	T	V	S	V	T	N	L	N	S	E	NEG
UK661	3	D	D	Y	A	I	G	I	T	N	I	S	S	Q	TDN
IBDVRF02-2017	3-1	D	D	Y	A	I	G	I	T	N	I	S	S	Q	NEG
IBDVRF06-2015	3-2	N	D	Y	T	I	D	I	T	N	I	S	S	Q	NEG
IBDVRF02-2016	3-3	N	D	Y	A	I	D	I	T	N	I	S	S	Q	NEG
IBDVRF04-2014	3-3	N	D	Y	A	I	D	I	T	N	I	S	S	Q	NEG
MG4	4	–	D	Y	S	V	S	V	T	T	L	N	S	Q	–
Mexico04M101	5	–	N	Y	T	V	N	V	T	N	L	N	K	Q	–
ITA-02	6	–	-	H	Q	V	S	K	S	N	L	S	S	Q	–
002-73	7	D	D	Y	P	V	G	V	T	N	L	S	S	Q	TES
IBDVRF05-2014	8	D	E	F	V	I	D	I	S	T	I	S	R	P	TDN
IBDVRF03-2017	Variant isolate	D	D	Y	T	I	D	I	T	N	I	S	R	Q	TDS
IBDVRF02-2018	Variant isolate	N	D	Y	A	I	D	I	T	N	I	S	S	Q	TDN

Identification of the reassortant viruses. Ten isolates in which the VP2 gene belongs to VV IBDV (Genogroup 3) are reassortants. In addition, Genogroup 8 isolates are also reassortants. The phylogenetic position determined by the analysis of the VP1 gene indicates that all three isolates are included in the VV IBDV group. Two variant isolates have VP1, also related to VV IBDV. Thus, 15 out of 28 tested VV IBDV isolates are reassortants.

Among isolates that do not belong to VV IBDV in terms of the structure of the VP1 gene, only the isolate IBDVRF07-2019 (Genogroup 1) is not a reassortant and has 99.36% homology with the vaccine strain D78 in both genes.

Isolate IBDVRF04-2014 (subgroup 3-3) has 100% homology with the vaccine strain 228E in the VP1 gene segment. The production of such a reassortant virus can be explained by the use of a vaccine based on the 228E strain.

An unusual group of reassortants was detected on the poultry farm of the Novgorod Oblast during 6 years. According to the structure of the VP2 gene segment, they are closely related (have 99% homology) and belong to subgroup 3-3. According to the structure of the VP1 gene, with the exception of IBDVRF01-2017, they are included in the endemic subgroup. The VP1 gene segment of the IBDVRF01-2017 isolate from the Novgorod Oblast has a high degree of homology with the STC strain. The origin of segment B of this isolate from a virus of a different genetic subgroup indicates that on this poultry farm there is a heterogeneous viral population, including IBDV from different genetic groups.

It has been shown that certain IBDV genetic forms can circulate on poultry farms for a long time. On the other hand, there is a change of virus isolates. So, on one of the poultry farms in the Kursk oblast, variant isolates IBDVRF03-2017, IBDVRF02-2018 were identified for 2 years, differing from each other in the nucleotide sequence of the hypervariable region of the VP2 gene by 4.7%. According to the structure of the VP1 gene segment, both isolates belong to VV IBDV and differ from each other by 1.8%.

Two different isolates IBDVRF06-2015 (Genogroup 3-2) and IBDVRF10-2019 (Genogroup 3-1) were detected on the poultry farm of the Yaroslavl Oblast. Both of them are reassortant viruses. The VP1 gene of these isolates belongs to different genetic subgroups: IBDVRF06-2015 belongs to the endemic subgroup, and IBDVRF10-2019 is closely related to the STC strain.

Previously, the degree of pathogenic action of three IBDV isolates was studied [29]. The study used the isolate IBDVRF08-2017, which has the VP1 and VP2 genes related to VV IBDV, and two reassortants: IBDVRF06-2019 characterized by VV VP2 and non-HV VP1; IBDVRF03-2017 with variant VP2 and VV VP1. It was found that in relation to the reference strain 52-70, the isolate IBDVRF08-17 (80.5%) had the highest virulence score. The least virulent isolates were IBDVRF06-19 (44.3%) and IBDVRF03-17 (43.9%).

Thus, the isolate, both genomic segments of which belonged to VV IBDV, showed the highest virulence. While reassortant viruses with only one segment related to VV IBDV showed a lower degree of pathogenicity.

CONCLUSION

Based on the analysis of the hypervariable region of the VP2 gene, the majority (22 out of 28) of the studied IBDV isolates belong to Genogroup 3 (VV IBDV). Three isolates detected in geographically close regions form a new Genogroup 8. Two isolates found on the poultry farm of the Kursk Oblast in 2017 and 2018 are variant. One isolate is a derivative of the D78 vaccine strain.

Only some of the isolates of Group 3 have VP1, which belongs to the VV IBDV. On the other hand, variant isolates and isolates of the new Genogroup 8 have VP1 characteristic of VV IBDV. Of the 28 isolates studied, 15 are reassortants. Among the reassortants, various combinations of genomic segments were identified (segment B – segment A): VV – endemic, VV – classical virulent, VV – attenuated strains, variant – VV, Genogroup 8 – VV. Identification of the diversity of combinations of genomic segments of IBDV indicates that a heterogeneous viral population circulates on poultry farms.

The study of the degree of pathogenicity of IBDV isolates showed that reassortant viruses are less pathogenic than VV IBDV.

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Contemporary issues in ensuring biological safety during disposal of biological wastes of animal origin by incineration in the Russian Federation

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SUMMARY
Animal management and breeding as well production, transportation, preparation, and processing of animal products and raw material result in generation of a considerable amount of biological wastes being a source of biological contamination of the environment and a clear threat to human and animal health. The animal biowaste incineration units are high threat facilities and require constant surveillance and control. Collection and analysis of data provided by the RF veterinary executive authorities were performed to objectively reflect the actual situation of the biological waste incineration facilities in the RF Subjects and to create a holistic view on the problem of interest in the country. The following parameters were analyzed: their number, type (stationary and mobile), type of ownership, location, availability of the certificate and highly-qualified specialists serving the biological waste incineration equipment as well as the availability of such facilities in the RF Subjects as for January 1, 2021. The analysis demonstrated that 4,459 biowaste incinerators were registered in the country. Most of these units are stationary and they belong to establishments involved in farm animal keeping, animal product processing, production and storing. Such equipment is mostly serviced by non-qualified staff ignorant of the technical characteristics and operating principles of this equipment. Almost one third of these units in the country are home made that is why their use does not guarantee complete destruction of biological wastes and pathogen inactivation. It was also revealed that the procedure for the incineration of biological waste of animal origin using home made incinerators is not legally fixed in the country. The results obtained show that the situation of the animal biowaste incineration in the Russian Federation is quite complicated.

Keywords: disposal, destruction, biological wastes of animal origin, animal biowaste incinerators, home made incinerator, cremating furnace, incinerators

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Актуальные аспекты обеспечения биологической безопасности при сжигании биологических отходов животного происхождения в Российской Федерации

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РЕЗЮМЕ

В процессе содержания и разведения животных, а также при производстве, транспортировке, заготовке, переработке продуктов и сырья животного происхождения образуется значительное количество биологических отходов, которые являются источником загрязнения окружающей среды и создают реальную угрозу здоровью человека и животных. Установки по сжиганию биологических отходов животного происхождения являются объектами повышенной опасности и требуют постоянного наблюдения и надзора. Для объективного отражения реальной ситуации с объектами сжигания биологических отходов в субъектах Российской Федерации и формирования целостного представления о рассматриваемой проблеме в стране был проведен сбор информации и проанализированы данные, предоставленные органами исполнительной власти субъектов Российской Федерации в области ветеринарии. Рассмотрены такие показатели, как количество, вид (стационарные, мобильные), форма собственности, расположение, наличие сертификата и квалифицированных специалистов, обслуживающих установки для сжигания биологических отходов животного происхождения, а также обеспеченность субъектов Российской Федерации данными объектами по состоянию на 1 января 2021 г. Анализ полученных первичных данных показал, что в стране зарегистрировано 4459 объектов сжигания биологических отходов животного происхождения, основная часть которых составляет стационарные установки, находящиеся в ведении предприятий, занятых содержанием сельскохозяйственных животных, а также переработкой, производством и хранением животноводческой продукции. В большинстве случаев обслуживание данных объектов осуществляет неквалифицированный персонал, который не владеет знаниями о технических характеристиках и принципах работы используемых установок. Почти треть установок по сжиганию биологических отходов животного происхождения в стране непромышленного изготовления, поэтому их использование не гарантирует полного сгорания биологических отходов и инактивации патогенов. Также выявлено, что в стране законодательно не закреплен порядок проведения сжигания биологических отходов животного происхождения в трупосжигательных печах. Полученные результаты исследования свидетельствуют о том, что в Российской Федерации сложилась напряженная ситуация в сфере сжигания биологических отходов животного происхождения.

Ключевые слова: утилизация, уничтожение, биологические отходы животного происхождения, объекты по сжиганию биологических отходов животного происхождения, трупосжигательные печи, крематоры, инсинераторы

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INTRODUCTION

A great number of biological wastes of animal origin including dead animals and birds, aborted and stillborn fetuses, condemned materials generated during veterinary and sanitary inspection at slaughterhouses, meat and fish processing plants, markets, sales facilities and other facilities are accumulated at establishments and backyard farms in the RF Subjects [1–4]. Decontamination of biological wastes of animal origin is critical for insurance of animal disease freedom in the country and its territories – RF Subjects [5, 6].

According to the RF law biological wastes of animal origin are decontaminated using three methods: disposal at rendering plants involved in meat and bone meal production; decontamination in biothermal pits; destruction by burning in animal biowaste incinerators, such as home made incinerators, cremating furnaces, incinerators. It is forbidden to bury the animal biowaste in the ground, throw into the trash cans, woods, ravines, water bodies as well as send to landfills or dumping grounds [1, 6–8].

Outbreaks of animal infectious diseases present serious problems for the State Veterinary Service of the RF Subjects. The key element of the successful response to the disease is the proper disposal and destruction of the dead animals and birds that died or were seized during the outbreak. Their appropriate and effective disposal can prevent and decrease the further pathogen spread including zoonosis agents [9–11].

Outbreaks of avian influenza, ASF and rabies have been reported during the last decade in the Russian Federation. Under conditions of the disease occurrence animal carcasses, biological materials and animal products contaminated with the disease agents shall be incinerated. Incineration of hundreds and sometimes thousands of carcasses is a complex technical task the solution of which requires the involvement of significant technical, human and financial resources.

Today two major methods of animal biowaste incineration are used.

The first one involves the use of special units that ensure high quality incineration of biological waste by equipping incinerators with special burners and afterburners, as well as an exhaust gas purification system and heat exchange equipment. The assembly and erection of these units take quite a long time, which is unacceptable under

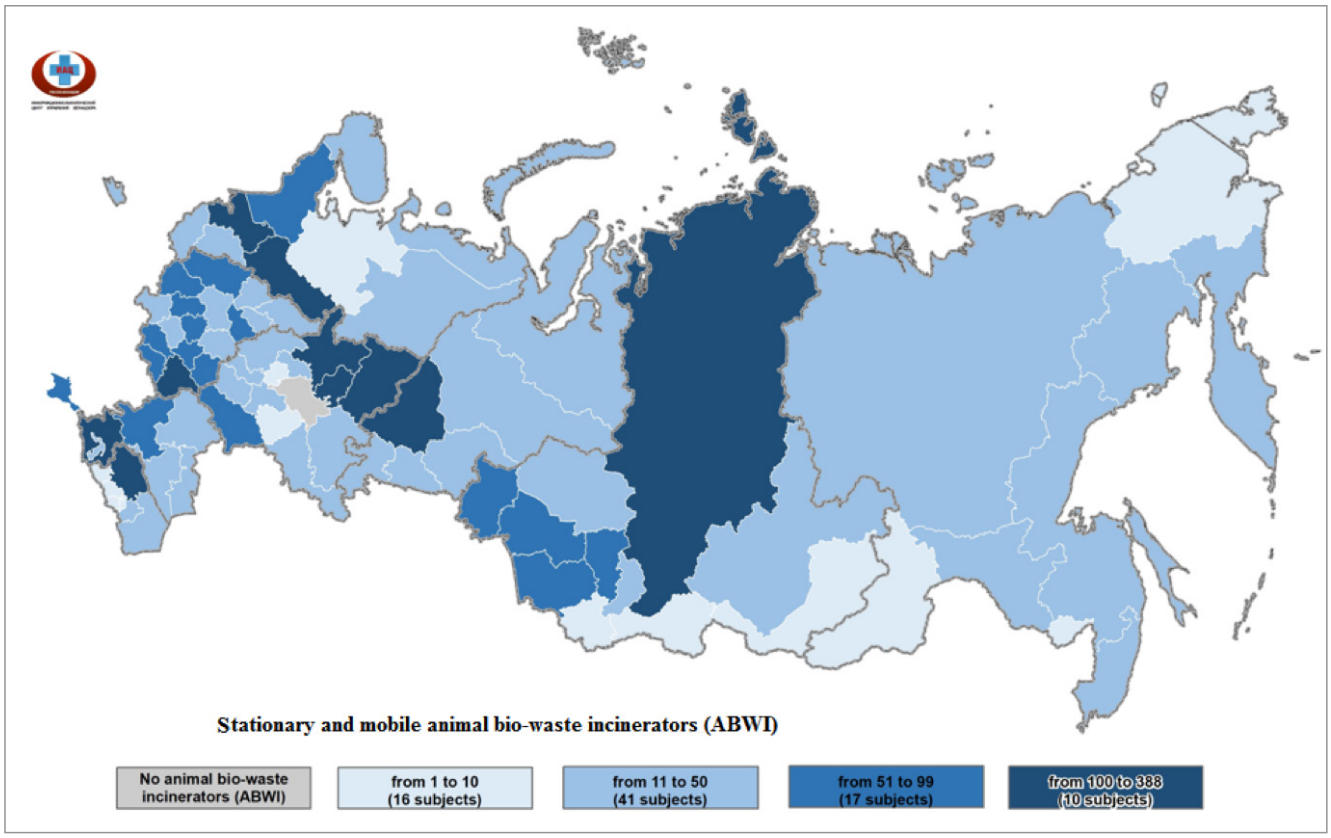

Fig. 1. Total number of animal biowaste incineration (ABWI) units registered in the Russian Federation (as of 01.01.2021)

epidemic conditions. Besides, work on such equipment requires professional training of maintenance personnel. Along with stationary units, there are mobile units that can be transported by almost any type of transport and installed on site in just a few minutes, but, as a rule, they have low productivity [12, 13].

The most common on the territory of the Russian Federation is the second method – the burning of animal biowaste in pits. The advantages of this method are obvious at first glance – relative simplicity and low costs. However, such an incineration method is ineffective, since the process of animal biowaste incineration using such a method is quite slow due to insufficient amount of oxygen in the burning area [14, 15]. The method of animal biowaste burning in pits should be gradually replaced by more contemporary methods, but actually it is still used in the countries with the high technological expertise.

In this regard the purpose of the research was to review the situation of ensuring biological safety in the country during animal biowaste incineration using especially designated units.

MATERIALS AND METHODS

In order to conduct the study, the specialists of the FGBI "ARRIAH" developed a form for collecting primary data, including such indicators as the number, type (stationary, mobile), ownership, location, availability of a certificate, and qualified specialists servicing animal biowaste incinerators, as well as the demand of Subjects for these units. Data for the period from 01.01.2020 to 01.01.2021 were collected using the Assol.Express operational reporting system. The primary data collected for 85 Subjects of the Russian Federation were analyzed.

The study used generally accepted methods of data analysis: generalization and formalization of information, the method of comparative analysis, methods of descriptive statistics.

In order to visualize the obtained data by mapping, the geographic information system ArcGIS 10.6 was used.

RESULTS AND DISCUSSION

General parameters. As of 01.01.2021, 4,459 animal biowaste incinerators are registered in the RF Subjects. They are located in all RF Subjects with the exception of the Republic of Tatarstan.

The total number of incinerators in the Russian Federation falls in the range of 2 to 388 units and most of them (45%) are located in 10 Subjects of the country. At the same time in 16 Subjects this parameter does not exceed 10 units, and in 41 Subjects of the country the total number of biological waste incineration facilities is in the range of 11 to 50 units (Fig. 1).

The results of the data analysis for the type of ownership revealed that 78% of the units are owned by or are on the balance sheet of organizations of various forms of ownership (SPK, OAO, ZAO, IP, etc.), whose activities are maintenance/rearing of farm animals, as well as processing, production and storage of livestock products. The smallest part of them falls on the municipalities of the RF Subjects (7.8%) (Fig. 2).

In the RF Subjects, the largest part (95%) of these units are stationary. The number of mobile incinerators

in the country is 231 units, which are registered in 44 Subjects. In most of them, the number of mobile biological waste incinerators is in the range from 1 to 7 units, while about half are concentrated in 5 Subjects.

In recent years, there has been a trend in the Russian Federation to reduce the number of animal burial grounds and biothermal pits. First of all, this is due to the introduction in 2006 of a ban on the destruction of biological waste by burial and the presence of a significant number of animal burial grounds not owned or managed by legal entities that would be responsible for their maintenance, preservation and use [3]. As a result, in some RF Subjects, targeted programs have been developed and put into effect. They are aimed at the elimination of unused and ownerless cattle burial grounds. The purpose of these programs is to prevent unauthorized burial of biological waste and the spread of pathogens of human and animal infectious diseases in the environment, as well as to reduce regional budget expenditures for their maintenance and ensuring their compliance with veterinary and sanitary requirements.

Thus, the municipalities of the RF Subjects, subordinate institutions of the State Veterinary Service of the RF Subjects, as well as livestock breeding and processing establishments are gradually moving from burial in animal burial grounds to disposal using animal biowaste incinerators (cremators, industrial and home-made incinerators). In the reporting period, the above institutions purchased and put into operation 266 incinerators, and 82% of them are organizations whose activities are related to keeping, raising farm animals, as well as processing, producing and storing livestock products.

Despite the fact that for the period from 01.01.2020 to 01.01.2021 the number of animal biowaste incinerators increased by 10%, in 44 Subjects of the Russian Federation, Subjects there is an additional demand for 2,129 incinerators. In some regions the number of units varies from 1 to 728 units, for 41% of the Subjects the demand for incinerators does not exceed 10 units.

Requirements for the animal biowaste incineration process in cremating furnaces. The disposal and destruction of biological waste of animal origin is regulated by the "Veterinary rules for movement, storage, rendering and disposal of biological waste", approved by the Order of the Ministry of Agriculture of Russia dated October 26, 2020 No. 626 [1].

These rules establish mandatory veterinary and sanitary requirements for the handling of biological waste of animal origin (collection, transportation, storage, disposal, destruction) both for animal owners, regardless of the method of farming, and for organizations and establishments of all forms of ownership.

However, in the veterinary legislation of the Russian Federation there is no any legal act regulating the procedure for animal biowaste incineration in home-made incinerators. There are no mandatory requirements for the technical characteristics of biological waste incinerators (mandatory certification, productive capacity, chamber volume, wall thickness, etc.), for their location (technical requirements for the room where these units are located and the enclosure), and for the incineration process itself (requirements for service personnel, disinfection of tools, transport and overalls, the method of destruction of the resulting combustion products, etc.) (Fig. 3).

The fact that this biowaste destruction method is not regulated, makes it difficult for the veterinary regulatory authorities to fully implement control and supervision measures for compliance with the veterinary and sanitary requirements for animal biowaste incinerators.

According to veterinary legislation, a veterinary specialist, after examining biological wastes of animal origin,

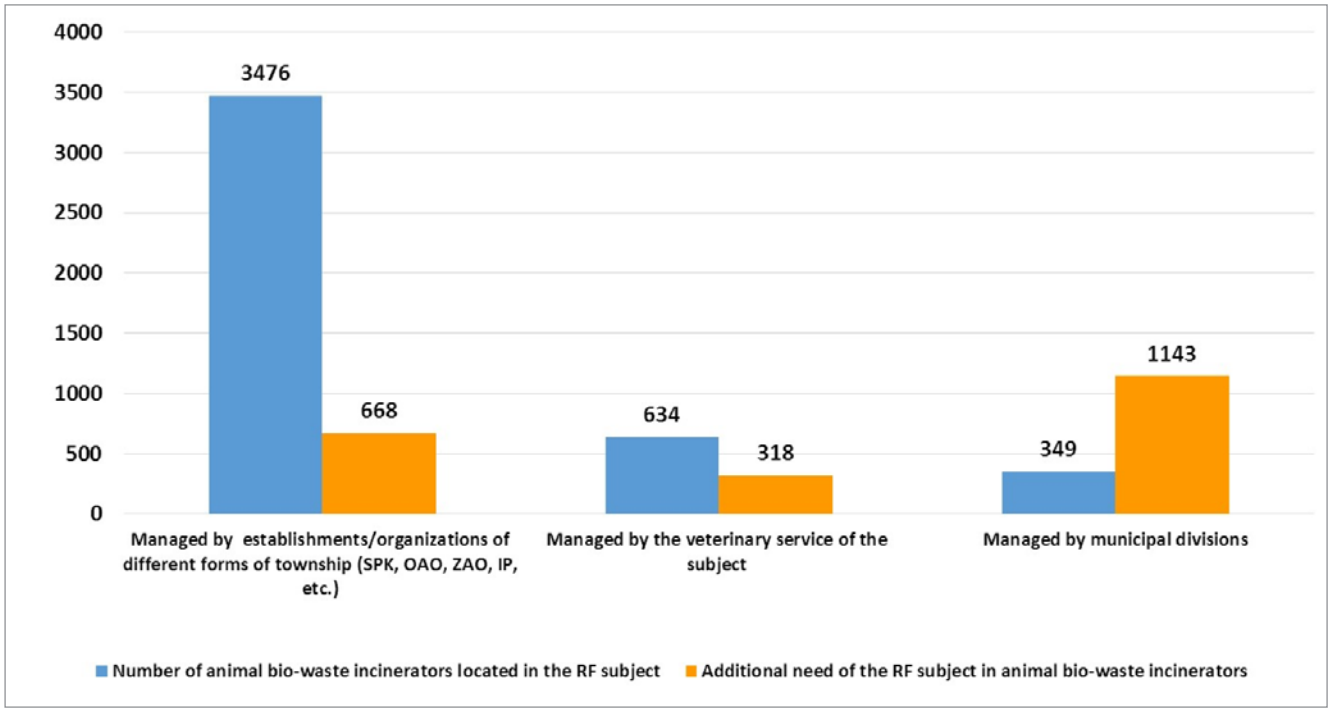

Fig. 2. Animal biowaste incinerator availability and demand in the country

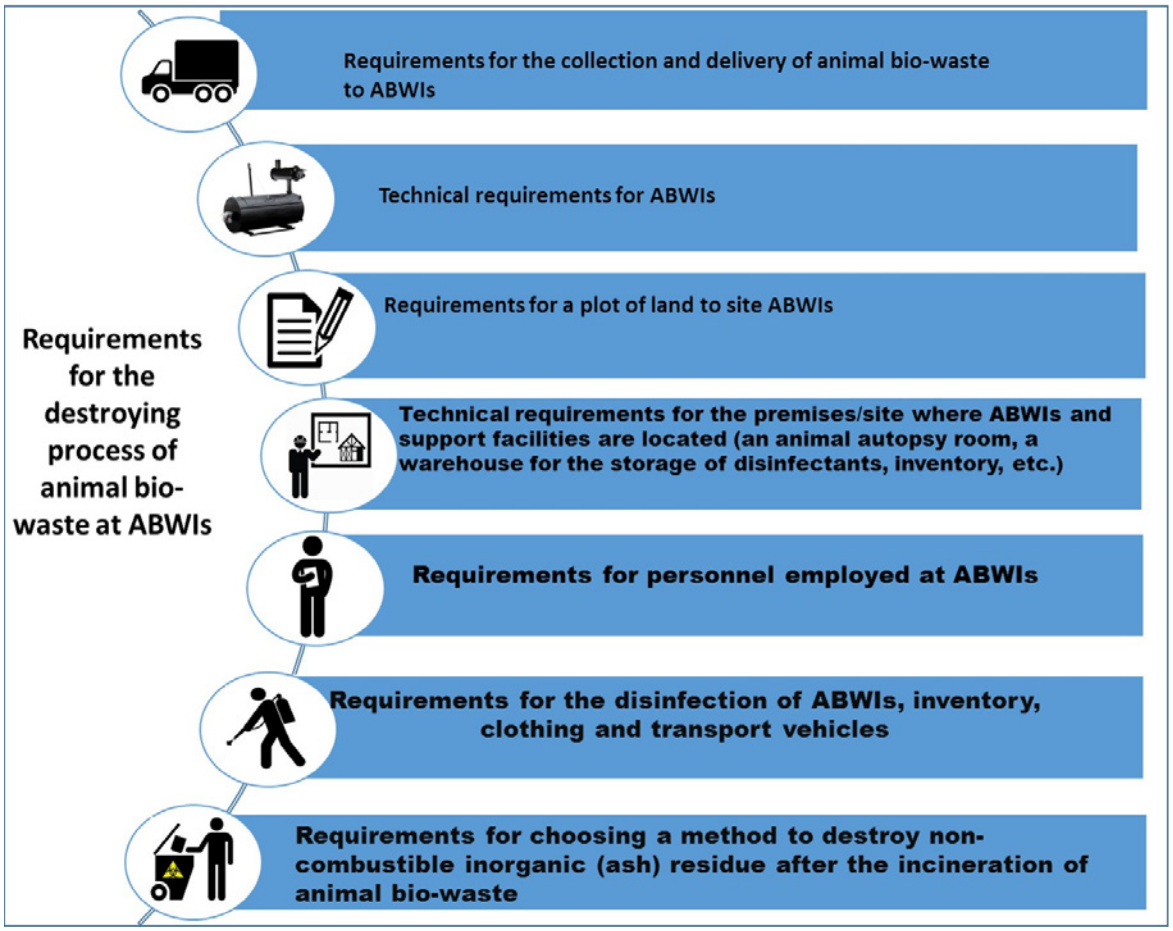

Fig. 3. Animal biowaste incineration procedure in home made incinerators, cremating furnaces and incinerators and its control

gives an opinion on their cleaning and decontamination method, and also prepares a veterinary accompanying document. Delivery of the biowastes to the place of destruction is provided by their owners. Biological wastes are placed in special closed containers and delivered by special transport means. Dead animals more 25 kg each, except for those contaminated with agents of anthrax, rinderpest, can be transported without biohazardous waste containers in vehicles covered with tilts or other devices preventing the dead animals from falling out and contamination of the environment [1]. This transport can belong to the owners of biowastes and organizations providing services for the destruction or disposal of biological waste of animal origin. At the same time, it should be noted that the containers and vehicles used for transportation of biological waste of animal origin are not subject to mandatory certification.

Since incinerators are high risk facilities, it is expedient to confirm that their quality complies with requirements for veterinary, sanitary, environmental and fire safety.

Currently, according to the Russian legislation, mandatory certification of biowaste incinerators is provided only for fire safety. Moreover, the certificate of conformity confirms the quality of not the entire unit, but only its component, in particular the burner used in it (gas, liquid fuel) [16]. The environmental certificate of compliance with environmental safety standards for manufacturers of biowaste incinerators is issued on a voluntary basis [17].

As for the certification of the animal biowaste incinerator conformity with requirements for veterinary and sanitary safety, this procedure is not provided for by the legislation of the Russian Federation. In this regard, it is not possible to determine the adequacy of the choice and observance of temperature and time modes for the destruction of various categories and types of biological waste of animal origin. Although these specific characteristics are the determining factor in the ability of biowaste incinerators to provide complete inactivation of animal pathogens.

Based on the practical experience of specialists who participated in the eradication of the infectious animal disease outbreak, it can be noted that the carcasses of various animal species burn for different periods of time and at certain temperatures. For example, turkey carcasses do not burn well enough compared to pig carcasses, which is due to the difference in the chemical composition and density of their muscle mass.

In the reporting period, 28% of the animal biowaste incinerators in the country do not even have fire safety and environment safety certificates of conformity. Thus, it can be assumed with a high degree of certainty that these units were not manufactured industrially, but are man made that is, any means at hand could be used for their construction: barrels, gas cylinders, welded boilers, etc.

The use of such units during biological waste burning cannot ensure the complete inactivation of infectious animal disease pathogens and, therefore, the proper level of biological protection of animals and humans from these pathogens.

According to article 7.1.12 SanPiN 2.2.1/2.1.1.1200-03 "On sanitary protection zones and sanitary classification of establishments, buildings and other facilities"[1], incinerators belong to Hazard Class I. In order to ensure the sanitary and epidemiological well-being of the population, a 1000 m buffer area should be established around biowaste incinerating facilities (sanitary protection zone). However, in 36 Subjects of the Russian Federation, incinerators (502 units) are located within the residential area. The bulk of these facilities are concentrated in the Central (37%) and Siberian (31%) Federal Districts.

It should be noted that compliance with this requirement is impracticable for the owners of mobile animal biowaste incinerators since the placement of these units on the territory of the settlement for destruction of biological waste is not regulated.

An essential condition for ensuring biological safety during the biowaste incineration is the correct arrangement of a room or building where animal biowaste incinerators and auxiliary premises are located: an autopsy room, a warehouse for storing disinfectants, tools, etc. At the moment, the RF legislation has no regulated norms and rules for these premises.

The incineration of biological wastes and the maintenance of the facility requires qualified personnel. It is necessary to allow persons who have been trained and tested on the technical characteristics, principles of operation and maintenance of incinerators to work with these units. In addition, the personnel must have a permit for the right to handle hazardous waste and be immunized in accordance with the national vaccination schedule.

During the research, it was found that 46% of the animal biowaste incinerators are served by personnel who have not undergone appropriate training. The lack of necessary knowledge regarding the technical characteristics of the units, as well as the process of incineration, disinfection, etc., can contribute to the spread of pathogens of infectious animal diseases into the environment.

Disinfection is critical when it comes to the system of veterinary and sanitary measures ensuring infectious animal disease freedom in the Russian Federation. Its main purpose is the destruction or neutralization of infectious animal disease pathogens (birds, fish, bees) in the environment [18].

In this regard, biowaste incinerators, tools, overalls, and vehicles used for transportation and burning of biological waste of animal origin shall be treated with disinfectants. Disinfection must be performed by trained specialists under the supervision of the State Veterinary Service. Employees responsible for disinfection also carry out sampling to control the quality of disinfection and maintain documentation (disinfection reports, disinfectant receipt, consumption logs, and a disinfection log). Disinfection quality control shall be performed after each biowaste incineration process.

Despite a significant decrease in the volume (up to 95%) of animal biological waste after incineration, combustion products (ash and other non-combustible residues) remain in incinerators, the destruction of which is carried out by various acceptable methods. During the reporting period the resulting combustion products after animal biowaste burning in most cases (63%) were taken to municipal solid waste landfills or dumped into biothermal pits, in 17% of cases they were buried at cattle burial grounds, and in 20% cases (in 31 RF Subjects) they were used as a fertilizer, which is a violation of Russian legislation, since this ash residue is not included in the list of certified fertilizers in the Russian Federation.

As noted earlier, almost a third of the available units in the country are home made, i.e. the use of these incinerators does not guarantee the complete combustion of biological waste of animal origin and the inactivation of pathogens. Consequently, the removal of residue combustion products to municipal solid waste landfills contributes to the contamination of territories with pathogens of infectious animal diseases.

CONCLUSION

The results of the analytical study performed show that as of January 1, 2021, there are a number of gaps in the field of animal biological waste incineration due to both the imperfect regulatory framework and the shortcomings in the organization of this system in the RF Subjects.

First of all, this is due to the fact that at the legislative level there are no rules regulating the procedure for animal biowaste incineration in home made incinerators. At the same time, the situation is aggravated by the fact that these hand made incinerators are used in a significant number of the RF Subjects, they have no certificates of conformity in the field of fire and environmental safety. About half of the incinerators are operated by unqualified personnel who are not aware of the technical characteristics and principles of operation of these units.

The current situation in the country in the field of ensuring biological safety during animal biowaste incineration using home made incinerators demonstrates the need to introduce the following corrective measures: fix the requirements for the technical characteristics of incinerators and the process of animal biowaste destruction at the legislative level; develop and adopt a legal act regulating the activities of mobile units for the thermal treatment of biological waste; develop unified forms for biowaste incinerator recording; prepare unified checklists for scheduled and unscheduled inspections of animal biowaste incinerating facilities performed by veterinary services; introduce mandatory certification of biological waste incinerators for their compliance with established veterinary and sanitary safety requirements.

It is also necessary to make additions to the existing "Cerberus" IS components of the "VetIS" information platform, which will allow the creation of the federal register of animal biowaste incinerators, whose veterinary and sanitary safety has been confirmed. This will allow the "Mercury" IS to block registration of veterinary accompanying documents for the movement of biological waste to disposal and destruction facilities not specified in this register.

[1] SanPiN 2.2.1/2.1.1.1200-03 "On sanitary protection zones and sanitary classification of establishments, buildings and other facilities": approved Decree of the Chief State Sanitary Doctor of the Russian Federation 25.09.2007 No. 74. Available at: https://base.garant.ru/12158477/b89690251be5277812a78962f6302560.

The implementation of the above measures will improve control over the safety of animal biowaste incineration in facilities intended for this particular purpose.

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