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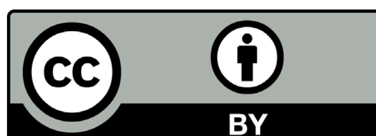

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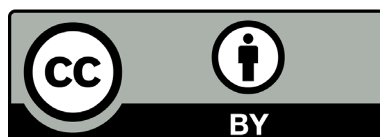

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EMERGENCE OF NEW INFECTIONS IN THE 21ST CENTURY AND IDENTIFICATION OF PATHOGENS USING NEXT GENERATION SEQUENCING

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Each new emerging infection may become a big challenge to the medical community. Changing environment, tropical deforestation, melting of the Antarctic ice, growing population density and uncontrolled use of antibiotics provoke emergence and evolution of pathogens. Epidemics caused by new strains of the influenza virus, respiratory syndromes associated with coronaviruses, outbreaks of hemolytic *Escherichia coli* infections and antibiotic-resistant superbacteria are hazards to humans. Among high-priority measures for pathogen control that are yet to be taken is development of fast and accurate techniques for pathogen identification. Our review looks at the cases of new infections registered in the 21st century and explores feasibility of next generation sequencing for the detection and identification of new pathogens.

Keywords: infectious agents, pathogens, techniques for identification of new pathogens, next generation sequencing, coronavirus, influenza virus, horizontal gene transfer, resistance to antibiotics

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ВОЗНИКНОВЕНИЕ НОВЫХ ИНФЕКЦИЙ В XXI ВЕКЕ И СПОСОБЫ ИХ ИДЕНТИФИКАЦИИ С ИСПОЛЬЗОВАНИЕМ ВЫСОКОПРОИЗВОДИТЕЛЬНОГО СЕКВЕНИРОВАНИЯ (NGS)

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Каждое новое инфекционное заболевание может быть серьезным вызовом для современной медицины. Изменение окружающей среды, вырубка тропических лесов, таяние льдов в Антарктике, увеличение плотности населения и повсеместное использование антибиотиков — все это факторы, провоцирующие появление новых патогенов. Эпидемии гриппа, вызываемые новыми штаммами вируса; респираторные синдромы, вызываемые новыми коронавирусами; вспышки инфекций, причиной которых является гемолитическая кишечная палочка; возникновение резистентных к антибиотикам супербактерий — примеры того, какие опасности могут поджидать человечество. В число первоочередных мер, которые необходимо предпринять для противодействия новым патогенам, входит разработка способов их быстрой и точной идентификации. В обзоре рассмотрены случаи возникновения в XXI веке новых инфекционных агентов, а также проанализированы возможность и перспективы использования для выявления и идентификации новых патогенов методов высокопроизводительного секвенирования (next generation sequence).

Ключевые слова: инфекционные агенты, патогенные организмы, методы выявления новых патогенов, высокопроизводительное секвенирование, коронавирус, вирус гриппа, горизонтальный перенос генов, антибиотикорезистентность

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Outbreaks of infectious diseases are a continuous threat to global health. A lot of effort is being put into the identification and study of new pathogens, among which are Middle East respiratory syndrome coronavirus, Zaire ebolavirus, and South American Zika virus. The table below lists factors that contribute to the emergence of new pathogens. However, a considerable proportion of epidemics are caused by known pathogens, such as poliovirus, influenza virus, or vibrio cholerae.

Risk factors contributing to the emergence of new diseases

Pathogen-associated factors
Accumulation of mutations in the genome
Horizontal gene transfer
Multipathogen infections
Changing antigenic determinants
Host-associated factors
Susceptibility to infections
Global transport networks
Increasing population density
Religious rituals and national traditions
Bioterrorism
Environment-associated factors
Changing environmental profile
Industrial development
Climate changes
Errors in healthcare
Animal epidemics
Wars and famine

Most outbreaks are caused by purely environmental factors, such as climate-related or geographical. However, human impact on the environment may also be a contributing factor. For example, some zoonotic diseases find their way into human communities because a natural habitat of their hosts has been destroyed. Aggravated by deforestation of mountain slopes, flooding causes outbreaks of cholera and other infectious diseases in populated areas. Some “anthropogenic” epidemics are directly linked to purposeful manipulations of pathogens. Modified in a lab, bioagents may be infectious or capable of acquiring virulence genes horizontally and therefore pose a serious biological threat. Mechanisms of new pathogens emergence are shown on Fig. 1.

Unfortunately, there are no thoroughly elaborated algorithms and ready commercial solutions to identify previously unknown pathogens. Techniques used to study their properties will vary in each individual case. The following review provides a detailed description of cases of emerging infectious agents of the 21st century and prompts a discussion about a possibility of elaborating a universal approach to pathogen detection using novel sequencing technologies.

New pathogens of the 21st century: examples and mechanisms of emergence

New coronaviruses

The 21st century has already seen the emergence of at least 9 new pathogens (Fig. 2). In 2002 the global healthcare was challenged by a previously unknown atypical pneumonia agent that came from China. In November 2002 a farmer died in the city of Foshan (Guangdong Province). Although the cause of death was inconclusive, it was clear that the patient had been afflicted with an unknown dangerous disease. On November 27, 2002 the Global Public Health Intelligence Network, a warning system developed by Health Canada in collaboration with the World Health Organization (WHO), picked up reports of an infection outbreak in China. Following a short investigation, WHO requested further information from China’s authorities. However, it was only after the epidemic crossed Chinese borders that details became available to the public. In February 2003 an American businessman died in Hanoi hospital after contracting pneumonia in China. The rate of disease progression was shocking. By March 15, the term “severe acute respiratory syndrome” (SARS) had been coined [1–3]; by March 27, its causative agent had been identified as a new coronavirus referred to as SARS-CoV [4–6]. From November 2002 to July 2003, a total of 8 098 patients in 25 countries contracted SARS; 774 patients died. In some populations [7] and age groups [8] mortality was as high as 40–55 %. Further scattered outbreaks of the infection were reported late in 2003 and early in 2004 in Singapore, Taiwan, Beijing and Guangzhou. All of them were linked to the cases of laboratory contamination and virus transmission from animals to humans [9], after the ban was lifted to sell palm civets in wet markets and serve palm civet dishes in restaurants imposed during the atypical pneumonia outbreak [10].

No effective antiviral agents were available at the time of the SARS outbreak [11], so basically, the treatment plan included supportive care and antibiotics to fight a secondary bacterial infection [12]. But due to the unprecedented international response, the outbreak was successfully contained [13]. Among the measures taken were contact tracing and isolation of people with suspected or confirmed SARS [14]. At present, SARS-CoV no longer circulates in the human population; however, a chance of a new epidemic remains as there are natural reservoirs of SARS ancestors, such as bats or other mammals [15].

Challenging as it was, researchers managed to identify the virus. Clinical specimens collected from patients with SARS were studied using cell cultures and molecular techniques. The virus was isolated in cell culture and then its 300-nucleotide-long RNA was detected by “random” polymerase chain reaction (PCR). Genetic characteristics of the virus revealed a very distant kinship to known coronaviruses (50 to 60 % similarity of nucleotide sequences). Based on the identified sequences, high sensitivity PCR- and real-time PCR-based assays were designed for virus detection. The virus was found in the clinical specimens of patients with SARS while the control samples came out negative. The sputum of infected patients was also found to contain high concentrations of viral RNA (up to 100 million molecules per 1 mL). Very low RNA concentrations were detected in blood plasma of infected patients in the acute phase of the disease and in their excrements by the end of treatment [4]. In spite of the fact that SARS outbreak was contained, SARS-CoV was not the only pathogen to threaten humans in the 21st century.

In 2003 a 7-month old baby presented to a hospital with obstructive bronchitis and conjunctivitis. A few tests were run to establish the presence of respiratory viruses, but all of them came out negative. A group of researchers headed by Lia van der Hoek proposed a modified technique for virus discovery based on cDNA-amplified fragment length polymorphisms (Virus-Discovery-cDNA-AFLP, VIDISCA). This method employs reverse transcription PCR (RT-PCR) with subsequent partial

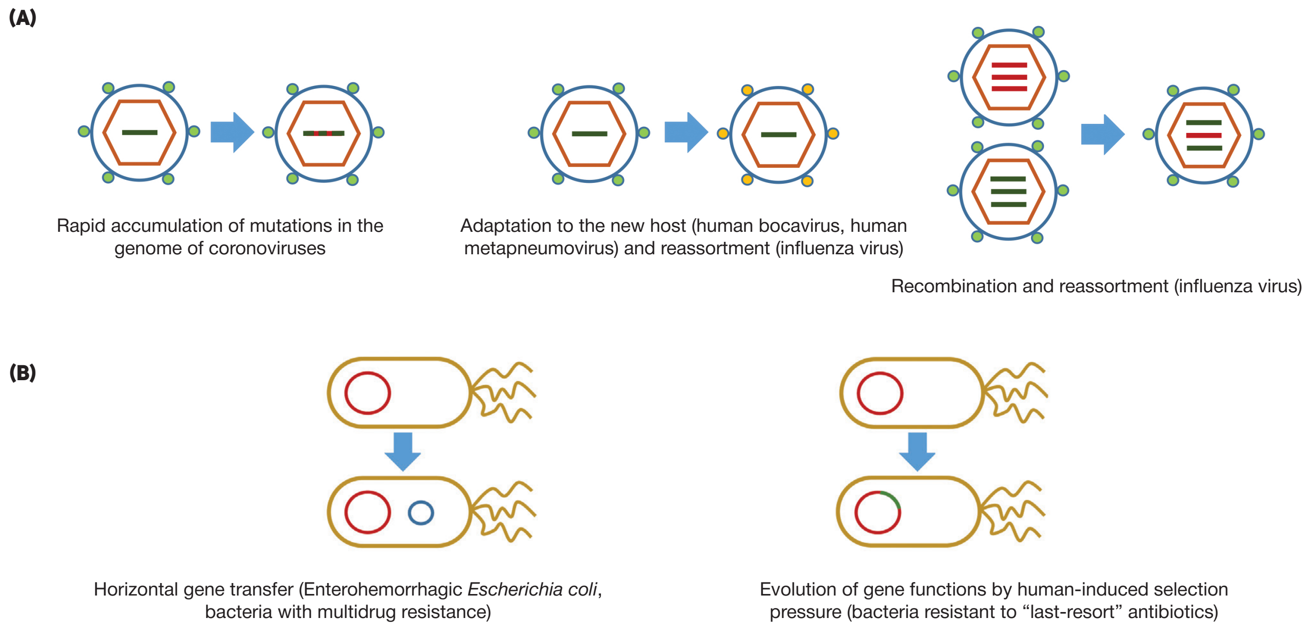

Fig. 1. Genetic mechanisms of emergence of new pathogens among viruses **(A)** and bacteria **(B)**

cDNA digestion by frequently cutting restriction enzymes. The assay results revealed a certain similarity of the discovered sequences to the sequences of the already known coronaviruses; however the difference between them was still sufficient to classify the studied coronavirus as new. Later, the virus was termed "human coronavirus NL63" [16].

In January 2004, a 71-year old patient from China presented to a hospital with pneumonia. Attempts to replicate the virus in cell cultures, RT-PCR and direct antigen tests of nasopharyngeal aspirates showed the absence of known respiratory viruses in the patient. RT-PCR performed to target a conserved region of the coronavirus polymerase gene confirmed the presence of a coronavirus but attempts to culture it failed. Partial sequencing of the viral genome showed that its sequence was highly homologous to the sequences of other βCoV viruses including HCoV-OC43, but had a different origin. This human coronavirus referred to as HCoV-HKU1 was later isolated from the aspirate of another female patient [17]. Shortly thereafter, the virus was cultured in human ciliated respiratory epithelial cells, but on the whole its replication in cell culture still remains a difficult task. Since its discovery, HCoV-HKU1 has been proved to occur worldwide, and the retrospective analysis of stored nasopharyngeal swabs confirms that it can be traced back at least to 1995 [18].

In June 2012 the world became aware of the existence of a new strain of a human coronavirus. A 60-year old patient was suffering from a severe respiratory infection at Dr. Soliman Fakeeh Hospital in Jeddah, Saudi Arabia. Standard tests could not identify the pathogen. Patient's sputum samples were sent to Rotterdam (Netherlands) where the virus was identified as a new coronavirus and termed HCoV-EMC (human coronavirus from Erasmus Medical College). The patient died later from acute pneumonia followed by kidney failure [19]. Since the discovery of the pathogen, a few of its isolates have been reported in the literature, various databases or mass media under different names. To study the virus, a research group was formed consisting of virologists whose major interest was in coronaviruses. To avoid confusion, the virus was given another name: the Middle East Respiratory Syndrome Coronavirus (MERS-CoR), which was approved by its discoverers, WHO and Ministry of Health of Saudi Arabia [19].

From June 2012 to February 7, 2014 there were 182 cases of MERS registered, of which 79 were lethal. According to WHO, by June 11 2014 there had been 699 laboratory confirmed cases; 209 people died [20]. Statistical reports reveal a 3-fold increase in disease prevalence over 4 months meaning that the epidemic is still raging. Mortality rates of up to 30 % are especially high in patients with comorbidities; patients with immunodeficiency or other primary diseases are also susceptible to the infection [21, 22]. There is also a serious risk of nosocomial transmission [23].

Clinical manifestations of MERS are similar to those of the acute viral respiratory infection and include such common respiratory symptoms as cough, fever and gastrointestinal dysfunction [24] before the onset of pneumonia [21]. Patients with MERS also tend to develop acute respiratory syndrome (ARS), renal failure, pericarditis and disseminated intravascular coagulation [24]. A risk of a pandemic is low since the virus is unlikely to effectively transmit between humans [24] and is transmitted only through close contact [25], between family members [26] or medical workers [27]; nosocomial transmission is also possible [28]. Patients with compromised immunity are especially susceptible.

The origin of MERS is not fully understood. Perhaps, the first transmission was from the camel to the human.

Over the past decade 4 new coronaviruses have been discovered, of which 2 are extremely dangerous; the other 2 were discovered accidentally and their signs are hard to distinguish from the signs of common acute viral respiratory infections. Our brief review shows that emergence of new highly virulent strains is very probable and only requires a couple of nucleotide polymorphisms in the viral genome to happen.

Human metapneumovirus

A new virus was isolated from the samples of 28 patients in the Netherlands in 2001. The symptoms of the infection were similar to those caused by the respiratory syncytial virus (RSV). A few patients were hospitalized; some required mechanical ventilation. Viral isolates were cultured in tertiary monkey kidney cells. Their cytopathic effect was pretty much identical to that of RSV. Electron microscopy of the supernatant of the infected

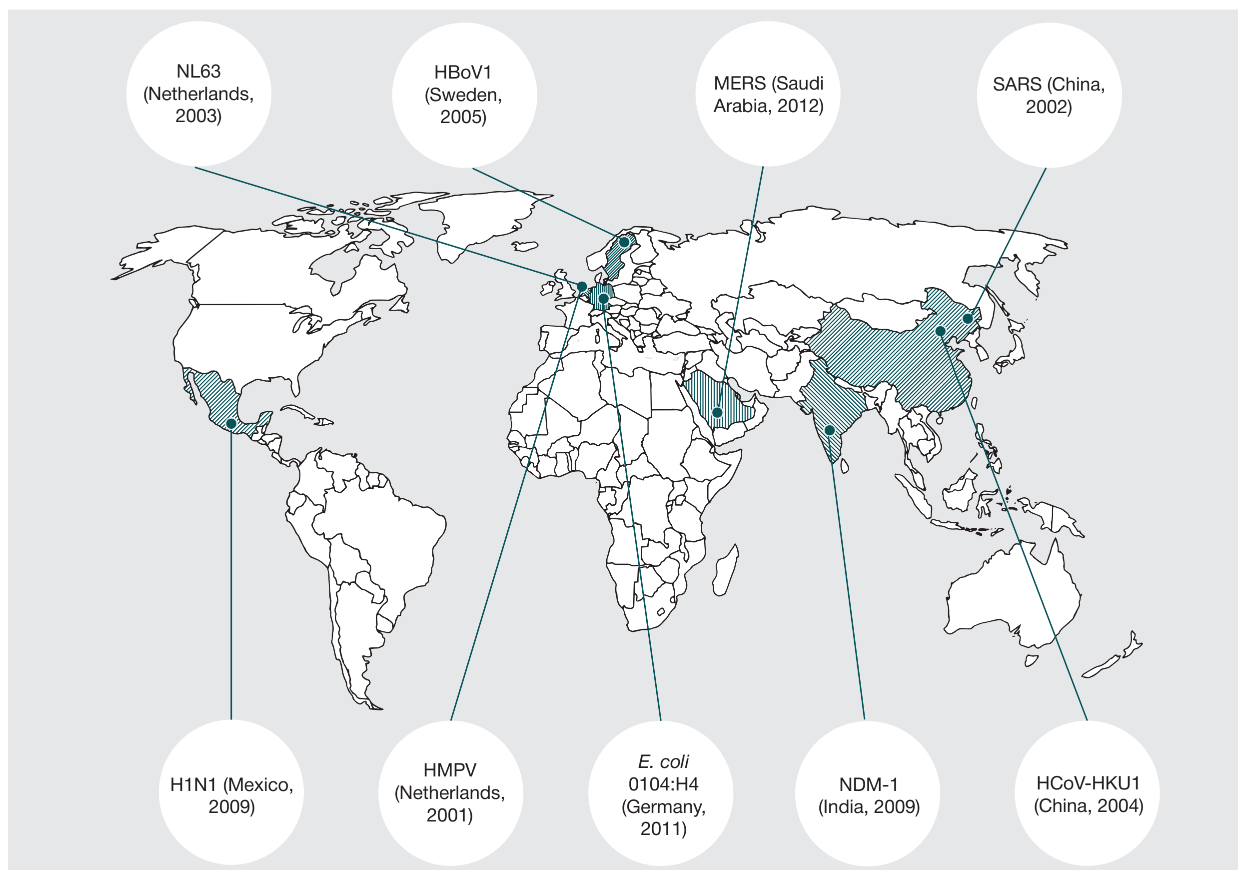

Fig. 2. Geographic distributions of emerging pathogens in the 21st century

cells detected paramyxovirus-like particles. But the use of real-time PCR primer sets for paramyxovirus detection yielded no results. Then a decision came to use RT-PCR assays with random primers to obtain information on the sequence of the unknown virus. Based on the similarity of sequences and genomic organization, it was concluded that the studied virus was a close relative of the avian pneumovirus. The virus was identified as a new member of the *Metapneumovirus* genus and called human metapneumovirus (HMPV) [29]. It was the first metapneumovirus capable of infecting humans. Although HMPV was discovered in 2001, phylogenetic analysis showed that the virus had been circulating in the human population for the last 50 years or so [30, 31]. From 7 to 19 % of respiratory infections in children who received either inpatient or outpatient care were caused by HMPV [32–34]. The literature reports that this virus ranks second in frequency among the respiratory viruses [35].

Human bocavirus

The first human bocavirus (hBoV) was discovered in 2005 in nasopharyngeal aspirates of 282 Swedish patients with the unknown infection of the lower respiratory tract. To remove all contaminating RNA from the samples, the latter were treated with DNAase prior to conducting RT-PCR with random primers. Bioinformatic analysis of obtained sequences revealed the presence of a new parvovirus in the samples that was highly homologous to bovine and canine parvoviruses (hence the name *Bocavirus*). The new virus was given a name of hBoV1 [36]. Three other strains of hBoV were discovered in 2010 and are now referred to as hBoV2, hBoV3 and hBoV4 [37–39].

HBoV1 causes respiratory diseases and is present everywhere across the globe accounting for about 19 % of all viral infections of the upper and lower respiratory tract in humans [40–42]. HBoV1 effectively infects epithelial cells of human airways and induces their cytolysis [43–45]. These data are confirmed by clinical observations indicating that the infection manifests as a respiratory condition. In contrast, hBoV2, hBoV3 and hBoV4 colonize the gastrointestinal tract; hBoV2 and possibly hBoV3 are associated with gastroenteritis [46, 47]. Interestingly, hBoV2 is the only intestinal bocavirus isolated from a nasal swab; therefore it may be associated with respiratory diseases [48, 49]. Though hBoV1 is found in all age groups, it is prevalent in infants of 6 to 24 months old [50, 51] and rare in adults [52–56]. Generally, transmission and infection occur throughout the year but are more often in winter and spring [55, 57–59].

Influenza virus

Another mechanism of pathogen evolution is genome recombination. A typical example here is a highly variable human influenza virus (IV) with a segmented RNA-genome. When several strains invade a host, their RNA segments may reassort to produce new pathogenic strains. Adaptive changes occurring in two surface proteins (hemagglutinin and neuraminidase) of the virus determine its ability to cause pandemics.

Water birds are a natural reservoir of IV in which the virus has evolved into its current state through several adaptation stages. Incredible diversity of IV strains is found in anseriformes and charadriiformes, including 17 hemagglutinin and 9 neuraminidase subtypes [60]. Transmission of the virus to land birds and mammals has triggered its rapid evolution [61]. Some strains of IV circulate in human populations (H1N1, H3N2), pigs (H1N1, H1N2), horses (H3N8, H7N7) and dogs (H3N8) [62]. Pigs have become a major reservoir for the pandemic strains

of the virus because they have receptors for both avian and human IV (2,3-sialic acids and 2,6-sialic acids, respectively) [63, 64]. Pigs are effective “mixing tanks” for the virus, a source of new reassortants that have mixed (recombinant) genomes and can cause another pandemic [61].

Pandemics are the most severe manifestation of the infection, with a 20–40 % global prevalence rate. One of the first documented IV pandemics occurred in 1918 when the deadly Spanish influenza took lives of 25 million people worldwide [60]. It was followed by the Asian flu (H2N2) in February 1957, Hong-Kong flu (H3N2) in 1968, Russian flu (H1N1) in 1977 and swine flu (H1N1) in 2009. The latter became the first and so far the last pandemic of the 21st century. H1N1 emerged through reassortment between the Eurasian swine influenza strain and North American triple reassortant H1N2 [65, 66]. In comparison with its “evil” ancestor, it is less virulent; however, it still caused 200,000 and 83,000 deaths by respiratory and cardiaovascular complications, respectively [67].

Since the discovery of a new H7N9 strain of avian influenza on March 30, 2013, China’s authorities have reported 135 laboratory confirmed cases of infection, with 45 deaths in Shanghai, Anhui, Jiangsu and Zhejiang [68]. The only case registered outside China was in Taiwan; however, the patient contracted the virus in China [69]. Those were the first cases of transmission of H7N9 avian influenza to humans [70, 71]. Initially, nonfatal viral infections caused by H7 strains (H7N2, H7N3, H7N5) were observed across Europe and in the USA [72]. The only exception in terms of fatality was a death case of H7N7 infection reported in 2003 in the Netherlands [73, 74]. Interestingly, those outbreaks occurred at the time of the flu outbreaks in poultry, but no such pattern was observed for H7N9. Cases of H7N9 infection seem to be epidemiologically unrelated, but the possibility of virus transmission between humans remains [75]. Delayed serologic response in patients infected with H7N9 complicates detection of the virus by serologic tests [76]. Besides, unlike H5N1, H7N9 infection in poultry tends to be latent, which makes identification of its source and a route of transmission much harder and increases a risk of a pandemic.

Shiga toxin-producing Escherichia coli

Another mechanism contributing to the emergence of new pathogens relies on the acquisition of new properties by an organism, such as an ability to produce toxins or resistance to antibiotics. A consequence of such genetic transformation was an epidemic caused by the O104:H4 strain of the enterohemorrhagic Escherichia coli in 2011 in Germany. It was the most severe outbreak ever registered caused by shiga toxin-producing *E. coli* (STEC): in total, 3 842 cases were reported including 2 987 cases of laboratory confirmed gastroenteritis (with 18 deaths) and 855 cases of hemolytic uremic syndrome (with 35 deaths) [77]. The outbreak started on May 8, reached its peak on May 22 and was over on July 4. The outbreak may have been halted because people had been warned against using contaminated food; however, delivery of contaminated products to markets may have also stopped. Allegations about the source of the infection were publicly debunked (at first cucumbers and cabbages were thought to be contaminated, but that was not true) [77]. On June 10, German authorities announced that infection had come from Egyptian sprouts of fenugreek [78].

Epidemiologic analysis of the infection initially transmitted through food is hard to perform once a pathogen learns to transmit between humans. Human-to-human transmission of enterohemorrhagic Escherichia coli O157:H7 was observed in about 20 % of households with an infected patient who had contracted the virus through food [79]. Secondary household transmission of the O104:H4 strain between adults was also observed in France [80] and the Netherlands [81]; it became possible due to the delayed onset of the infection compared to the standard incubation time (7 to 9 days for O104:H4). Secondary transmissions were observed in Hessen (Germany) that lied outside of the epidemic area in the North [82]. Investigations proved the facts of household and nosocomial transmissions; there was also a case of transmission between laboratory staff.

Within a very short time, the O104:H4 strain isolated in Germany was sequenced by a few groups of researchers. The first sequence was obtained in the Beijing Institute of Genomics from a sample provided by the University of Hamburg. Expedited by the use of the Ion Torrent platform, sequencing of the bacterial genome only took 3 days. The first annotated sequence was published by researchers from the University of Goettingen who used the following genome sequencers: Flex [83], Ion Torrent [84] and PacBio RS [85]. A combination approach based on the used of several next generation sequencing techniques yielded higher assembly quality (longer read lengths, fewer errors and missed regions, etc.). Sequence mapping revealed a similarity between the studied strain and 4 other strains of enterohemorrhagic Escherichia coli that had also caused infection outbreaks, including enteroaggregative *E. coli* (EAEC) isolated from AIDS-stricken patients with chronic diarrhea in the 1990s in Central Africa [86]. However, the African strain did not contain the Stx2 prophage [84]. Mellmann et al. proposed a model of O104:H4 evolution according to which the progenitor strain had transformed into O104:H4 by removing or acquiring mobile DNA elements through horizontal transfer [83]: a German variant of the pathogen had acquired plasmids that carried fimbriae/pili genes (AAP/I) and lost plasmids that carried the genes of TEM-1 and CTX-M-15 enzymes responsible for developing resistance to antibiotics. Comparison of the epidemic strains also revealed extensive rearrangements in the isolates, including deletions, insertions and inversions, which indicated considerable genomic mobility. Researchers also found that it was those structurally different regions that contained fragments encoding virulence factors.

Why was strain O104:H4 so virulent? The study of genome and virulence genes showed that this strain had an unusual combination of SPEC virulence genes (prophage Stx2, long polar fimbriae, tellurite resistance, iron metabolism) and EAEC virulence genes (AAF/I, transcriptional regulator AggR, dispersin Aap and shigella enterotoxin Set1) [87]. The latter are localized to the pAA virulence plasmid [83]. Thus, the virulence of the O104:H4 strain is ensured by two different mobile elements, prophage Stx2 and plasmid pAA, which is quite unusual. It may have been the combination of SPEC and EAEC virulence factors that shaped this new extremely dangerous pathogen. It causes cytotoxic damage to the intestinal epithelium facilitating systemic absorption of shiga-toxin, which may explain the high prevalence of hemolytic uremic syndrome in Germany. But in spite of 2 antibiotic-resistance genes in O104:H4, the epidemiologic situation, in particular mortality rates, could have been worse if the virus had had resistance to a broader range of antibiotics.

Antibiotic resistance and superbacteria

Since the first cases reported in the 1980s, strains with multidrug resistance (MDR) have become common sources of

nosocomial infections [88]. Many countries, including Russia, have increasingly witnessed infections resistant to traditional antibiotic treatments. It should be noted that major sources of infections caused by such pathogens as methicillin-resistant staphylococcus aureus, vancomycin-esistant enterococcus and other gram-negative bacteria with MDR are intensive care units [89].

Carbapenem resistance of gram-positive bacteria poses a particular problem. Carbapenems are drugs of choice used to treat many infections caused by gram-negative bacteria [90]. Extensive use of carbapenems promoted antibiotic resistance in bacteria. The most common carbopenem-resistant microorganisms are *Pseudomonas aeruginosa*, *Acinetobacter baumannii* and *enterobacteria* [91].

Pseudomonas aeruginosa causes acute invasive infections in patients with compromised immunity or in critical condition. Isolates of *P. aeruginosa* obtained from patients of intensive care units demonstrated resistance to carbopenems in 28–37 % of cases [92, 93]. *A. baumannii* is also one of the major sources of nosocomial infections. Initially this pathogen was sensitive to imipenem treatment in most medical institutions. But soon its strains were rapidly evolving to develop carbopenem resistance. At the moment 50–60 % of nosocomial infections associated with *A. baumannii* do not respond to imipenem treatment [94, 95]. Many enterobacteria, a broad range of beta-lactamase-producing *E. coli* and strains of *Klebsiella pneumoniae* resistant to carbopenems pose a serious threat to patients in intensive care because carbopenems are used as last resort antibiotics [96].

The driving force of carbopenem resistance is thought to be the extensive use of the third generation cephalosporins, aztreonam and ipinem. Emerged in the 21st century, superbacteria are totally resistant to any known antibiotics and are a serious challenge to modern medicine. Emerging pathogens are a product of both acquired resistance genes and the activation of "hidden" resistance genes resulting from a few significant nucleotide polymorphisms. Such genetic modifications are typical for microorganisms. In this light, a focus on the bacterial resistome — a sum of all resistance genes in the entire microbial community — is a prerequisite for effective identification and elimination of pathogens.

The resistome concept is based on the fact that soil actinobacteria and many other microorganisms actively produce antimicrobial compounds. It seems obvious that in order to survive, a microorganism not only has to develop defense against antibiotics: it also needs an ability to produce them. As proved by some studied, many resistome components emerged long before antibiotics were introduced into clinical routine [97]. Metagenomic analysis of ancient DNA samples collected in permafrost zones revealed the presence of beta-lactam-, tetracycline-, and glycopeptide-resistance genes [98]. It was shown that modern glycopeptide-producing organisms harbor ancient glycopeptide resistance genes (vanHAX). Moreover, the VanA protein, one the most important products of glycopeptide resistance genes, has preserved its function and 3D structure over centuries [99]. In another study, bacteria found in caves that had had no contact with the surface for over 4 million years proved to be resistant to 14 different antibiotics [100]. Genotyping and biochemical assays show that resistance genes are present in the microbial pangenome regardless of the human-induced selective pressure [100].

Although the independent ancient origin of antibiotic resistance genes is evident, humans have largely contributed to the formation and transformation of the resistome in its current state. Resistance protogenes do not form a stable phenotype but are capable of transforming into resistance genes when undergoing a mutation or due to contextual changes. Mutations of the enzyme facilitating its transition from one functional class to another are highly unlikely to occur while the expansion of the substrate specificity range in the enzymes with retained function is very probable. Structural studies demonstrated the evolutionary proximity between lincosamides and aminoglycoside nucleotidyltransferases and polymerases, and this allows for a supposition that progenitor polymerases were resistance protogenes that later evolved into antibiotic-modifying genes [101].

Conserved structural elements and biochemical mechanisms detected in a similar way indicate that protein kinases and protein acetyltransferases share common ancestors with resistance protogenes from which aminoglycoside resistance genes were derived [102, 103]. Moreover, resistance genes themselves can function as resistance protogenes. For example, aminoglycoside acetyltransferase acc(60)-Ia-cr ensures resistance to quinolones [102]. The ancestral enzyme acc(60)-Ia ensures resistance to kanamycin (which is an aminoglycoside); mutations of its two amino acid residues Trp102Arg and Asp179Tyr turned to be sufficient to extend its substrate specificity to include a number of quinolone antibiotics, such as ciprofloxacin, without losing aminoglycoside acetyltransferase activity.

The frequency of resistance protogenes in the resistome is unknown. To be considered clinically significant, these protogenes have to undergo a series of important evolutionary events. However, the examples above show that enzymes have a potential to include more substrates in their "profile" and might contribute to the emergence of new resistance genes.

Similar to resistance protogenes, silent resistance genes cannot form a resistant phenotype in their current structural state. Unlike protogenes, these genes can be detected in the resistome based on the homology between their sequences and the sequences of known resistance genes. For example, two antibiotic-sensitive strains of *Citrobacter freundii* isolated before antibiotics entered the clinical setting contain AmpC beta-lactamase genes [103]. Mutations that trigger AmpC expression in these strains induce resistance to broad-spectrum cephalosporins. The wild type of *Salmonella enterica* cultured in the enriched growth-supporting medium is sensitive to streptomycin and spectinomycin. However, the same strain is resistant to both drugs when cultured in nutrient-poor medium due to the activation of aminoglycoside adenyltransferase gene aadA [104]. Overexpression of aadA from a plasmid resulted in streptomycin resistance (the minimum inhibitory concentration of streptomycin increased). Thus, a total expression level of a resistance gene may be critical in the formation of a resistant phenotype.

If a mutation is seen as a driving force of evolution, then horizontal gene transfer is a magic wand that can transform the inactive resistance gene into a fully functional one by increasing the number of gene copies or changing the context that ensures gene expression under a strong promoter. Having become a component of a mobile element, resistance genes discover an opportunity to spread throughout the entire microbial pangenome where they can pick up further mutations reinforcing their function and expanding the range of possible enzyme substrates in response to the environmental selection pressure.

Staphylococcus aureus with its variety of genes capable of horizontal transfer in human pangenome is a perfect illustration of their role in antibiotic resistance: mobile elements account for 15–20 % of its genome, including

bacteriophages, pathogenicity islands, plasmids, transposons, and staphylococcal cassette chromosome mec [105]. Accumulation of these mobile elements is a result of selection pressure, but the element source is bacteria that once co-existed with *Staphylococcus aureus*. While details of interactions between pathogens and commensual bacteria remain largely unclear, we are coming to realize that major reservoirs of resistance genes available to pathogens are harbored by the human microbiome [106]. Thus, metagenomic libraries that include samples of intestinal microbiomes of infants, children and teenagers report resistance to 14 antibiotics [107]. Moreover, all libraries report resistance to tetracycline, trimethoprim, trimethoprim sulfamethoxazole, D-cycloserine, chloramphenicol, and penicillin, and some of them report resistance to aminoglycosides, glicylcyclines and beta-lactams. About 3 % of all antibiotic resistance genes listed in those libraries are associated with mobile elements, such as transposons or integrons [108]. The effect of antibiotics on intestinal microbial communities is actively studied. For example, some antibiotics, especially metronidazole and beta-lactam, negatively affect the variety of microorganisms in the gastrointestinal tract [108]. If any bacterial taxon starts to dominate the gut flora, it increases a risk of bacteremia [109].

Members of the human gut flora can acquire resistance genes horizontally (from farm animals to humans through food). A group of researchers discovered that 42 unique resistance genes had been transmitted to the human microbiome by agricultural isolates, which allows for the assumption that the microbial flora of farm animals, as well as waste, may contribute to the development of drug resistance in human pathogens [109]. Mobile elements that carry antibiotic resistance genes are widely spread in the microorganisms we consume with food [109–113] and are a potential source of resistance genes for the human microbiome. Unfortunately, overuse of antibiotics on farms is not rare. Monitoring on Chinese pig farms [114] showed that antibiotic resistance genes were found almost everywhere in the soil, as the latter was fertilized with the manure of pigs who had received antibiotic-containing food. Tests of pathogen-containing agricultural samples revealed a 3-fold increase in the number of unique resistance genes compared to the controls, including resistance to clinically significant antibiotics, such as macrolides (mphA and erm), cephalosporins (bla-TEM and blaCTX-M), aminoglycosides (aph and aad) and tetracycline (tet). The number of transposases in the genomes of pathogens found in pig manure and soil samples was 90 000 and 1 000 times higher, respectively, than in the controls. The number of transposases positively correlates with the frequency of resistance genes (especially tetracycline resistance genes) in the microbiome of agricultural products.

To sum up, all mechanisms of emergence of new pathogens can fall into two categories:

1. host-to-host transmission of the known pathogen accompanied by an acute infection in the new host due to the lack of adaptation of the latter (a good example here is a cytokine storm);
2. emergence of new pathogenic properties in the known biological agents usually acquired through horizontal gene transfer.

Identification of new pathogens using traditional methods. Difficulties

So far, a lot of technologies and commercial applications have been developed for pathogen detection and identification. They can “spot” nucleic acids and antigens typical for a pathogen. Although many of those methods are claimed to meet the strictest requirements for sample preparation, processing rate, accuracy and reliability, only a few of them can be used in real life circumstances, especially in the field [115]. Biohazard detection systems must ensure timely identification and confirmation of biological risk factors straight in the sample yielding as few false positive or false negative results as possible. Such systems must be able to detect a modified or an unknown pathogen. Devices for biohazard detection must be portable, easy to use and capable of detecting several or even dozens or hundreds of factors simultaneously [115].

Currently there are a few diagnostic methods that meet most of the listed requirements but there is not a single tool that would meet all of them. Unlike chemical detectors capable of scanning a sample for health-threatening amounts of chemical compounds, low-sensitive biological detectors rarely “spot” potentially hazardous amounts of pathogens straight in the sample; what is more, the sample must be preprocessed before the test. Diagnostic systems based on nucleic acid amplification are generally more sensitive than antibody-based systems [115]. For example, PCR assays can detect individual molecules of microbial nucleic acids within a relatively short time [116–118]. However, this technique still requires thorough preparation of the sample and cannot directly detect toxins or infectious agents deprived of nucleic acids (such as prions) [115].

Specificity is a no less important parameter of a diagnostic method, as there is always a need to minimize background signals or false positive results when processing a complex mix of organic and inorganic compounds. High levels of competitor antigens or DNA fragments in the sample may render the test nonspecific. High sensitivity of PCR-based assays may actually be their drawback in the case of contaminated samples yielding false positive results due to the presence of various substances, including humic acids and heme, that inhibit polymerase activity.

Another important requirement for a diagnostic method is its reproducibility, which may be influenced by a number of factors, including reagent stability or varying test conditions. The impact of these factors may be reduced by introducing standards for sample collection and subsequent analysis.

In addition to the requirements listed above, diagnostic methods must be capable of performing a multiplex analysis, i.e. detect more than one bioagent in a sample. Samples often contain a mix of toxins, bacteria, viruses, etc. Besides, there may also be genetically or antigen-modified elements, previously unknown microorganisms or emerging strains of well-known pathogens, all of which are extremely difficult to detect. It should be noted that even regular bioagents are hard to detect in contaminated samples. Human specimens (blood or excrements), food, water, or air samples are “difficult” objects for diagnostic systems. For example, anticoagulants, leukocyte DNA or heme components inhibit PCR [115, 119, 120], which leads to false negative results. Fat in food samples and concomitant bacteria in excrements may distort immunoassay results. Therefore, biological agents must be isolated or purified before the analysis, which means longer tests and renders field diagnostics impossible.

Sample composition determines conditions for its storage and transportation. Air and water samples must be brought to concentrations allowing preliminary detection of target molecules. Air samples must be transformed to a liquid state because the majority of diagnostic tests work with liquids. Sample volumes and transportation are also important especially when it comes to living organisms. Sometimes to

assess the risk, the viability of a pathogen must be confirmed; in this case standard genetic or immunological assays will be of no use.

Over the past years, methods for detection and identification of unknown pathogens have been actively developed and profusely funded [115]. The most promising technology among them is next generation sequencing.

Next generation sequencing. Basic principles

The term "next generation sequencing" (NGS) is used to describe a group of methods for parallel sequencing of multiple fragments that unlike Sanger sequencing allow reading massive volumes of primary DNA sequences in one go. NGS has become a truly universal method of describing genomes of living organisms. Currently NGS-based applications are actively used in scientific research, molecular systematics, bioengineering, cellular and molecular biology, and in routine human activities: medical practice, criminology, selection, etc.

There are two major groups of NGS types: sequencing of multiple preamplified DNA fragments and single-molecule sequencing.

All sequencing methods based on template amplification share the same principle regardless of the reagents or devices used. First, a library is prepared by DNA fragmentation and adapter ligation. Then, library fragments are immobilized on beads or flow cell surface; each fragment is amplified by emulsion bead PCR or bridge PCR, respectively. Specific primers are then hybridized to adapter sites and sequencing is performed. This process is accompanied by signal emission. Signal type depends on the platform used. The signal is registered by the device that converts it into a nucleotide sequence.

Pyrosequencing or 454-sequencing was a pioneer NGS variation. The idea behind it is as follows: when a nucleotide is added to an elongating complementary sequence, light is emitted. [121–123]. Another NGS type, semiconductor sequencing, is based on measuring changes in pH values caused by H^+ release that occurs during formation of phosphodiester bonds as nucleotides are added to a complimentary strand [124–127]. Another NGS variation is sequencing by ligation: a sequencer captures a fluorescent signal emitted during complimentary strand synthesis in a flow cell into which a mixture of fluorescently labeled nucleotide probes (octamers) and a DNA ligase are pumped [128]. The most common type of NGS is sequencing by synthesis which employs fluorophore labeled reversible terminator nucleotides. Amplification is performed inside a porous flow cell into which reagents for DNA synthesis are pumped [129]. After cluster PCR amplification, clusters of clonal DNA copies are generated to the cell surface, with each cluster corresponding to one read. High cluster density (up to 800–900 thousand per mm2) provides sufficient throughput in terms of the obtained data. Clusters of DNA molecules are then sequenced according to the principle similar to the Sanger method [130, 131].

Among the drawbacks of NGS based on the use of preamplified DNA fragments are sequencing errors in homopolymer regions or regions that contain single nucleotide polymorphisms; problems related to repeat resolution; dependence of read accuracy on GC-content of DNA fragments, etc. [132–134]. All of these factors dictate the need for alternative sequencing techniques, such as single-molecule sequencing.

One of its types is based on the use of DNA polymerase to catalyze incorporation of a fluorescently labeled nucleotide into the elongating strand. Incorporation is captured by a highly sensitive CCD-camera. Once the nucleotide is incorporated, the fluorescent label is removed and fluorescence goes back to normal values. Then another nucleotide enters the DNA polymerase active site and the cycle is repeated [135]. Phage φ29 DNA polymerase used in this technique of can process up to 10 nucleotides per second. This technique can be used to sequence long DNA molecules — up to 10 000–20 000 base pairs and already has a number of practical applications [136–142].

Another type of single-molecule sequencing uses electrophoretic cells equipped with a nanopore membrane. Single stranded DNA molecules are threaded into the pore; as the molecule enters the pore, the amount of current that passes through it changes [113]. Based on the properties of this change, such as duration and amplitude, it is possible to accurately identify the nucleotide that enters the pore at a particular time point. So far, this approach has been implemented in one commercial sequencer (MinION by Oxford Nanopore Technologies, UK) distributed under the early access program [143]. The advantage of this sequencing type is a possibility to run long reads without having to use expensive equipment. Its major drawback is high error rates (12–20 %) [144]. However, it is becoming clear that nanopore sequencing is an increasingly promising technique for metagenomic studies, sequencing of short genomes, identification of viral and bacterial agents. Nanopore sequencing has been successfully used as a diagnostic test to detect Ebola and Chikungunya viruses. It is also a good technique for conducting metagenomic research of bacterial resistome and sequencing large-scale genomes [145–147].

NGS-based strategies for pathogen identification

The main group of pathogens that pose the biggest threat to humans includes bacteria and viruses. Other pathogens such as fungi or protists are no less dangerous but do not normally require a genetic analysis to be identified. At present, the major technique for describing a diversity of microorganisms in the sample is metagenomic analysis. It has become possible and even routine due to NGS. The diversity of microorganisms found in the sample can be described using two different strategies: targeted sequencing of selected marker regions and large-scale (whole) metagenome sequencing.

The first method is simple, cheap and takes less time for sample preparation, sequencing and data processing. However, it has its limitations and can only be used to detect the presence of different organisms in the sample. In contrast, the second method yields a full profile of the microbial community, including the description of its genetic properties. Usually regions of the 16S rRNA gene of prokaryotes and 18S rRNA gene of eukaryotes are recommended as marker regions for processing metagenomic samples; for fungi samples ITS regions are recommended [148–150]. However, the task may dictate the use of other markers. For example, for generating a resistome profile, regions of antibiotic resistance genes should be selected.

The second method is costly and time consuming. However, whole genome sequencing provides a basis for further assembly of a reference genome [148]. These are three ways to analyze the obtained data (fragments of microbial DNA contained in the studied metagenomic sample). The first method involves comparison of marker sequences with known sequences obtained from databases that describe genomes of similar organisms [151–153]. The second method involves

clustering of all reads into taxon groups (based on their similarity to known whole genome sequences, etc.) [154–157]. The third method is based on the assembly of the obtained contigs into genes or even genomes *de novo* [158, 159]. Whole genome sequencing and methods of data interpretation are highly useful tools for pathogen identification as they help to identify individual genes in the sample.

Both approaches have their own drawbacks and advantages. Sequencing of individual regions is fast and cost-effective and gives a general idea of the genetic diversity of the sample, while whole metagenome sequencing provides full information on pathogen determinants (metaresistome etc). Most of the obtained data will not be of any particular value but what is important is that sequencing will yield a comprehensive list of genetic elements that determine epidemiologic properties of pathogens contained in the sample. It might be possible to use a reagent kit instead of conducting whole genome sequencing that consists of several hundreds of oligonucleotide sequences complementary to important epidemiologically significant determinants. The use of the kit would speed up metagenomic processing and cut its costs. Then total/whole genome sequencing could be used in some difficult cases following preliminary pathogen detection to provide a detailed genetic profile of a mixed sample or isolated pathogen.

Specific aspects of NGS-based identification of viruses and bacteria

State-of-art NGS techniques are ideal when there is a need to analyze, identify and describe genomic sequences of isolated prokaryotic organisms. NGS certainly holds promise as an effective tool for identification of unknown pathogens in mixed samples. However, there may be a difficulty in detecting horizontal transfer factors in samples containing prokaryotic organisms; drawing up accurate genomic profiles for individual members of such microbial communities may also be an issue. To minimize these issues when working with chromosome-bearing genetic elements, a better coverage of chromosome sequences of individual metagenomic components by single reads is required.

Quality of data obtained through sequencing is largely determined by sample preparation. Its significance becomes obvious once we take a closer look at the aspects of virus identification. Identification of new viruses is a challenging task: viral nucleic acids are very hard to isolate from junk nucleic acids. Extraction of nucleic acid from virus particles obtained through ultrafiltration of large DNA viruses results in sample contamination by the so-called gene transfer agents: nonviral DNA packed in viral capsids [160]. Identification of small highly variable RNA viruses is complicated by the presence of contaminating amounts of rRNA in nucleic acid samples. There are certain difficulties with primer selection: primers need to be universal and allow amplification of at least genus-specific viral cDNA [161]. One of the popular techniques used to identify emerging viruses relies on a modified PCR assay (VIDISCA) followed by NGS (Fig. 3) [162]. Below is a brief description of the technique.

First, the sample is selectively enriched with viral nucleic acid; as part of the procedure, the sample is centrifuged to remove residual cells and mitochondria. The sample is also treated with nucleases to remove interfering chromosomal and mitochondrial DNA and RNA from lysed cells. Adding RNAsa to the sample causes degradation of cellular RNA, but the viral nucleic acid remains intact, because it is packed inside a capsid. Then nucleases are inactivated and viral nucleic acids are extracted from the sample. RNA is reverse transcribed into cDNA and a complementary strand is synthesized from viral RNA or genomic DNA [163]. Double stranded DNA is then digested by frequently cutting restriction enzymes (HinP1-I and Mse-I). The cleaved DNA is then ligated to Hinp1-I and MseI adaptors with complementary overhangs. Target molecules are amplified using primers specific to each adaptor. For further selective amplification primers with a supplementary base (G, A, T or C) are used. In total, 16 combinations of primers are used; each sample is compared to the negative control (uncontaminated serum or plasma and supernatant of noninfected cultures). PCR products specific for infected samples are then cloned and Sanger-sequenced.

This technique is quite difficult to perform and its throughput is relatively low; reproducibility may also be an issue [163]. Currently, a modification of the method is attempted based on a combination of PCR with NGS. The amplified fragments are conjugated to nanoparticles and sequenced by massive parallel sequencing. The original method was based on the pyrosequencing technology; the license for it was acquired by Roche. But a serious problem arouse related to a low number of clean reads due to the presence of ribosomal RNA (rRNA) in the sample. Therefore, the method yielded poor results. There are a few approaches that can help to reduce the amount of

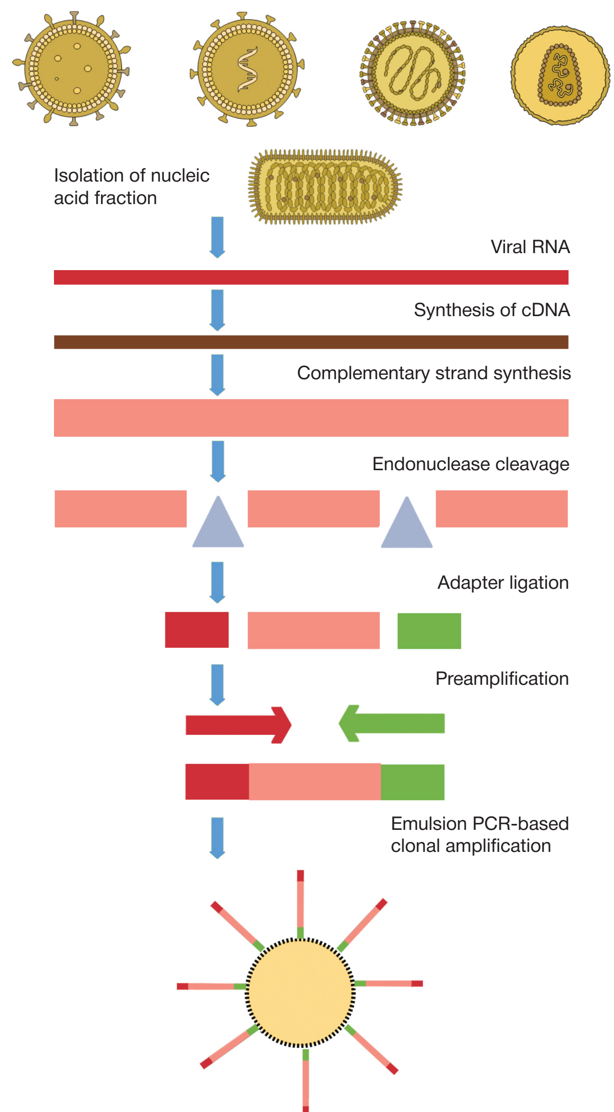

Fig. 3. Identification of new viruses using VIDISCA and next generation sequencing

contaminating rRNA in the samples such as the use of specially designed primers that do not anneal to rRNA, low-frequency-cleavage restriction enzymes and specific oligonucleotides for blocking cDNA synthesis on rRNA [163]. Although these “patches” significantly reduce the number of amplified rRNA fragments, the obtained result is still far from being perfect, as viruses are detected in only 50 % of contaminated samples. However, if the problem of rRNA removal from the samples is fully solved, the technique will certainly be one of the most time-saving and accurate tools ever used for the detection of previously unknown viruses.

CONCLUSION

Emergence of new bacteria and viruses that pose a serious threat to global health is inevitable and dictated by evolution. Viruses and bacteria are highly adaptive due to a number of molecular mechanisms at their disposal, such as recombination, reassortment and horizontal gene transfer. Coupled with a capacity to produce abundant progeny and human-induced selection pressure, these mechanisms expedite emergence of new pathogens. Considering close international contacts among humans, pathogen spread to new areas aggravating the risk of epidemics. However, this risk may be reduced by the development of new methods for infection control (vaccination, medications, new sterilization technologies), and techniques for pathogen identification that must take into account the genetic adaptive capacity of pathogens. Literature review revealed that there are no ready commercial solutions for identification of organisms with new pathogenic properties. Traditional PCR and immunoassays have a number of limitations. One of the most promising methods used to identify a broad range of pathogens is next generation sequencing.

Next generation sequencing is one of the few available methods that can detect a pathogen, generate its genetic and epigenetic profile, and provide information on the microbial community inhabiting the sample. Rapid evolution of sequencing techniques makes the analysis easier, cheaper and faster. Enhanced with a variety of software applications, next generation sequencing becomes an effective tool for identification of previously unknown pathogens.

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GENETIC POLYMORPHISM OF *STAPHYLOCOCCUS EPIDERMIDIS* STRAINS IN PATIENTS OF THE NEONATAL INTENSIVE CARE UNIT

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Staphylococcus epidermidis is a member of the normal bacterial flora of humans capable of causing potentially dangerous diseases in neonates with very or extremely low birth weight. The number of genes responsible for virulence and antibiotic resistance may vary in different *S. epidermidis* strains. We sequenced isolates of *S. epidermidis* to explore genetic diversity of 14 strains circulating in the Neonatal Intensive Care Unit of Kulakov Research Center for Obstetrics, Gynecology and Perinatology. Among the studied strains, 8 sequence types were identified, the most frequent being ST2 and ST59, both of which belong to the clonal complex CC2. Of 14 studied strains, 10 were of CC2 type. The studied strains revealed a variety of genes responsible for antibiotic resistance. We found 15 genes that provided resistance to aminoglycosides, beta-lactam antibiotics, fusidic acid, macrolides, lincosamides, streptogramin B, tetracycline, and trimethoprim. We identified a number of genes associated with virulence (*aae, atlE, aap, embp*), whose frequency in the studied isolates was varied. The insertion element *IS256* was detected in 9 strains, and 7 strains revealed the presence of the *ica*-operon responsible for the biosynthesis of the biofilm matrix proteins.

Keywords: polymorphism, NGS, virulence, resistance, *Staphylococcus epidermidis*, neonates, sequence type

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ГЕНЕТИЧЕСКИЙ ПОЛИМОРФИЗМ ГОСПИТАЛЬНЫХ ШТАММОВ *STAPHYLOCOCCUS EPIDERMIDIS*, ВЫДЕЛЕННЫХ У НОВОРОЖДЕННЫХ ОТДЕЛЕНИЯ РЕАНИМАЦИИ И ИНТЕНСИВНОЙ ТЕРАПИИ

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Staphylococcus epidermidis — представитель нормальной микрофлоры человека, способный вызывать опасные заболевания у новорожденных с очень низкой и экстремально низкой массой тела при рождении. Различные штаммы *S. epidermidis* обладают разным спектром генов, ассоциированных с их резистентностью к антимикробным препаратам и патогенностью. По результатам анализа данных полных геномных секвенирований четырнадцати штаммов *S. epidermidis* изучено генетическое разнообразие штаммов, циркулирующих в отделении реанимации и интенсивной терапии новорожденных НЦАГиП им. В. И. Кулакова. Выявлена принадлежность штаммов к восьми сиквенс-типам, из которых чаще встречались ST2 и ST59, принадлежащие к единому клональному комплексу CC2. К данному клональному комплексу относились 10 из 14 штаммов. Показано, что изученные штаммы обладали широким спектром генов резистентности к антимикробным препаратам. Обнаружено 15 различных генов, обуславливающих резистентность штаммов к аминогликозидам, бета-лактамным антибиотикам, фузидиевой кислоте, макролидам, линкозамидам, стрептограмину B, тетрациклину и триметоприму. Выявлены гены, ассоциированные с патогенностью (*aae, atlE, aap, embp*), встречающиеся в геномах изученных штаммов с разной частотой. В геномах девяти штаммов был обнаружен инсерционный элемент *IS256*, а в геномах семи штаммов — гены *ica*-оперона, отвечающего за синтез белков матрикса биопленок.

Ключевые слова: полиморфизм, полногеномное секвенирование, патогенность, резистентность, *Staphylococcus epidermidis*, новорожденные, сиквенс-тип

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Staphylococcus epidermidis is a member of the normal flora inhabiting human skin and mucosa [1, 2]. Still it can cause life-threatening diseases, such as pneumonia, sepsis and catheter-related infections, in neonates with low and extremely low body weight. The pathogenic potential of these bacteria varies over a very wide range, from virulence to commensalism. In spite of advances in our understanding of pathogenic mechanisms used by *S. epidermidis*, they still remain understudied.

S. epidermidis strains have different genetic properties. A method of multilocus sequence typing (MLST) has been developed and is actively used to classify bacterial strains into sequence types. Although the number of *S. epidermidis* sequence types is quite big, the majority of the nosocomial strains from around the world can be grouped to form a single clonal complex (CC2) that comprises closely related sequence types [2, 3]. Many of *S. epidermidis* strains circulating in the hospital environment are resistant to methicillin; this type of antibiotic resistance is associated with mobile genetic elements referred to as staphylococcal cassette chromosome mecs (SCCmec) [1, 4]. Resistance to methicillin is mediated by the *mecA* gene that encodes an alternative penicillin-binding protein with reduced affinity to methicillin (oxacillin). Methicillin resistance is a pressing clinical issue due to the fact that *mecA* of staphylococcal strains confers resistance to all beta-lactam antibiotics (penicillins, including protected penicillins, cephalosporins and carbapenems). Gene sets contained in SCCmec elements may vary and include genes encoding resistance to other antibiotics, as well as insertion sequences, such as *IS431*, plasmids, such as *pT181*, and transposons, e. g. *Tn554*.

Nosocomial strains of *S. epidermidis* also tend to be resistant to aminoglycosides and macrolides and, to a lesser degree, to tetracycline, chloramphenicol, vancomycin and clindamycin [5]. According to some researchers, methicillin (oxacillin) resistance is observed in over 70 % of *S. epidermidis* nosocomial strains [6, 7]. Antibiotic resistance genes are often found in mobile genetic elements of *S. epidermidis*. On the whole, bacterial strains demonstrate a strong correlation between the presence of resistance genes and resistance phenotypes [1, 8].

S. epidermidis strains harbor a few virulence determinants in their genome, i. e. genes encoding synthesis of proteins that promote infection and sustain bacterial viability. Although some works report the association between bacterial virulence and virulence factors, it is currently impossible to identify markers that could be used to accurately differentiate between pathogenic and harmless strains [1]. Perhaps, advances in this field depend on the study of complex interactions between bacteria and the immune system of humans. Some virulence factors are part of the core genome and can be found in all strains of *S. epidermidis*. Other factors associated with pathogenicity are harbored by few strains. One of the approaches used to identify genetic features of microbial strains relies on high-throughput whole-genome sequencing that allows performing a comprehensive analysis of genetic properties of selected strains and also detecting a large number of genes or their variants by conducting a bioinformatic analysis of the obtained assemblies. Here, in Kulakov Research Center for Obstetrics, Gynecology and Perinatology we study genetic diversity of various microbial strains that circulate in the hospital environment [2, 9].

The aim of this work was to study genetic diversity of nosocomial strains of *S. epidermidis* isolated from patients of the Neonatal Intensive Care Unit of Kulakov Research Center for Obstetrics, Gynecology and Perinatology.

METHODS

We isolated 14 strains of *S. epidermidis* from patients of the Neonatal Intensive Care Unit of Kulakov Research Center for Obstetrics, Gynecology and Perinatology. Strains 3, 7, 8, 13, and 14 were isolated from tracheobronchial aspirates of 5 neonates; strains 1, 5, and 9 were isolated from stool samples of 3 neonates; strains 4 and 10 were isolated from oropharyngeal swabs of 2 patients; strains 2 and 11 were isolated from blood samples of 2 neonates; strain 12 was isolated from eye discharge of 1 patient; strain 6 was isolated from the lung tissue of one neonate postmortem. All neonates had signs of infection (pneumonia, sepsis, conjunctivitis) at the time of sample collection; autopsy of the dead neonate confirmed pneumonia. All neonates had low or extremely low body weight.

Specimens were inoculated into Columbia agar base (Bio-Rad, USA) supplemented with 5 % sheep blood (Eco-Lab, Russia) and mannitol salt agar (Liofilchem, Italy). Plates were incubated in the thermostat at 36–37 °C for 48 hours.

Isolates were identified to a species level using matrix-assisted laser desorbtion/ionization time-of-flight mass spectrometry (MALDI-TOF MS). Sample preparation is described below. One-day old microbial cultures (1–2 colonies) were applied onto a stainless steel target plate, allowed to dry for 1–2 minutes, and then covered with 2 μL saturated matrix solution (a saturated solution of alpha-cyano-4-hydroxycinnamic acid by Bruker Daltonics, Germany, in 50 % acetonitrile and 2.5 % trifluoroacetic acid). All reagents including water were either analytical or mass spectrometry grade. Crystals were exposed to air for 5–10 min to dry. Humidity and temperature were not controlled. Two replicates were run for each sample. Mass spectrometry was performed using MALDI-TOF mass analyzer Autoflex III (Bruker Daltonics, Germany) equipped with a nitrogen laser of 337 nm wavelength. All measurements were performed in linear mode with positive ion detection. To accumulate mass spectra, laser power was set to a minimum threshold sufficient for sample desorbtion/ionization. Mass analyzer settings were optimized for the m/z range of 2,000 to 20,000. External calibration was performed using reference masses of known *Escherichia coli* proteins. The sample was applied onto 3 steel targets; the spectrum was recorded as a sum of 10 measurement runs, with 50 laser bursts per run. To record, process and analyze the spectra, flexControl 2.4 (Build 38) and flexAnalysis 2.4 (Build 11) software by Bruker Daltonics, Germany, was used. Mass accuracy was ± 2 Da. Cluster analysis and comparison of the obtained mass spectra with the values retrieved from mass spectrometry reference databases were performed using Biotyper 1.1 software (Bruker Daltonics, Germany).

Antibiotic sensitivity tests were performed on the automated microbial identification system Vitek 2 Compact (bioMérieux, France) according to the manufacturer's protocol. The samples were tested for their sensitivity to benzylpenicillin, cephoxitin, gentamycin, clindamycin, erythromycin, vancomycin and fusidic acid. Results were interpreted using the software supplied by the manufacturer based on the interpretation criteria recommended by EUCAST in 2015.

Genomic DNA was extracted from freshly grown cultures using lysozymes and protein kinase K. DNA was further purified by phenol-chloroform extraction. To obtain DNA libraries, we used Ion Xpress Plus Fragment Library Kit and Ion Xpress Barcode Adapters (Thermo Fisher Scientific, USA) following the manufacturer's protocol. Quality of the libraries was tested on Agilent 2100 Bioanalyzer system using HighSense DNA Kit

(Agilent Technologies, USA) according to the manufacturer's protocol. Emulsion polymerase chain reaction (PCR) and enrichment of ion sphere particles were performed using Ion OneTouch Template Kit (Thermo Fisher Scientific) according to the manufacturer's protocol. Sequencing was performed on the Ion PGM Torrent platform using Ion Sequencing Kit and 316 v1 chips (Thermo Fisher Scientific) according to the manufacturer's protocol. Short reads were assembled into longer sequences (contigs) using MIRA 3 software with the following parameters: job = genome, *de novo*, accurate.

Sequence typing was performed using MLST 1.8 software [10]. Polymorphism analysis of 7 loci (*arcC*, *aroE*, *gtr*, *mutS*, *pyr*, *tpi* and *yqiL*) of the assembled contigs was conducted. Antibiotic resistance genes were identified using ResFinder 2.1 software [11]. The minimally acceptable degree of similarity was 90 %, the minimal overlap length was 70 %. Virulence-associated genes and loci were identified by comparing their sequences to the corresponding nucleotide sequences obtained from the GenBank database [12] using BLAST software [13].

RESULTS

We isolated 14 strains of *S. epidermidis* from the patients of the Neonatal Intensive Care Unit of Kulakov Research Center for Obstetrics, Gynecology and Perinatology. Whole-genome sequence was run for each strain followed by sequence typing, identification and analysis of genes associated with virulence and antibiotic resistance. Results of genome assembly are shown in Table 1.

Sequence typing identified 8 sequence types among the strains. The most frequent types were ST2 and ST59 (each detected in 4 strains). The rest 6 strains belonged to types ST19, ST22, ST87, ST173, ST210, and ST218. All strains belonged to known sequence types.

A summary of antibiotic resistance genes identified in *S. epidermidis* is presented in Tables 2 and 3.

S. epidermidis strains were found to harbor amynoglycoside resistance genes, namely *aacA-aphD*, *aadD* and *aphA*. The most frequent of them was *aacA-aphD* detected in 12 strains (strains 1–12). Gene *aadD* was also present in the genomes of 5 strains (strains 1, 2, 4, 9, and 10), gene *aphA* — in the genomes of 2 strains (strains 3 and 7). These genes are believed to form a phenotype resistant to some antibiotics of the aminoglycoside family. According to the recent studies [8], locus *aacA-aphD* forms a phenotype resistant to amikacin, gentamycin, kanamycin and tobramycin. Gene *aadD* mediates resistance to amikacin, kanamycin, neomycin and tobramycin, while gene *aphA* mediates resistance to kanamycin and neomycin. A gentamycin resistance phenotype was observed in thirteen studied strains carrying resistance genes.

The studied strains were found to carry 2 beta-lactam resistance genes. The *blaZ* gene that encodes beta-lactamase and is capable of cleaving penicillins was present in the genomes of all studied strains. Phenotypically all strains were resistant to benzylpenicillin. Resistance to methicillin in staphylococci is determined by the *mecA* gene that encodes an alternative penicillin-binding protein with low affinity to methicillin [1, 14]. Gene *mecA* responsible for methicillin resistance was found in 13 of 14 strains (strains 1–12 and 14). Phenotypically, all strains demonstrated resistance to methicillin (cephoxitin), i. e., were methicillin-resistant.

Of 2 known genes that mediate resistance to fusidic acid, only one — *fusB* — was detected in the studied strains (strains 2, 4–6, 9, 19). Gene *fusC* was absent. Of 14 strains, 10 exhibited phenotypic resistance to fusidic acid.

Some researchers demonstrated that staphylococci can harbor *vanA* genes mediating resistance to vancomycin [8]. However, the strains studied in our experiments did not carry vancomycin resistance genes (*vanA*, *vanB* and *vanZ*), and none of the strains had a vancomycin-resistant phenotype.

Phenotypic resistance to lincosamides (clindamycin) was demonstrated by 7 strains. We identified 2 genes associated with lincosamide resistance. Gene *lnu(A)* was present in 4 strains (strains 1, 3, 8, 14) while gene *vga(B)* was found in strain 5 only. Gene *vga(A)* that can mediate resistance to lincosamides was absent in the studied strains.

Among the studied strains, there were 10 with phenotypic resistance to macrolides (erythromycin). Genomes of strains 6,

Table 1. Genome assembly of *S. epidermidis* strains based on whole-genome sequencing data

Strain ID	Number of contigs	Genome length, million b. p.	N50, b. p.	GC-composition, %
1	249	2.6	96 722	32.03
2	312	2.8	126 592	31.86
3	186	2.5	116 897	32.05
4	291	2.8	97 452	31.89
5	162	2.5	73 775	32.16
6	166	2.7	140 749	31.82
7	178	2.5	105 269	32.14
8	147	2.5	62 617	32.08
9	442	2.7	121 229	32.04
10	529	2.8	138 397	31.80
11	192	2.6	104 362	32.03
12	183	2.6	103 850	32.02
13	110	2.5	229 537	31.94
14	211	2.6	122 737	31.96

Table 2. Antibiotic resistance of *S. epidermidis* strains

Antibiotic	Strain /sample type/													
	1	2	3	4	5	6	7	8	9	10	11	12	13	14
	stools	blood	tracheobronchial aspirate	oropharyngeal swab	stools	lung tissue	tracheobronchial aspirate	tracheobronchial aspirate	stools	oropharyngeal swab	blood	eye discharge	tracheobronchial aspirate	tracheobronchial aspirate
Benzylpenicillin	R	R	R	R	R	R	R	R	R	R	R	R	R	R
Cephoxitin	R	R	R	R	R	R	R	R	R	R	R	R	R	R
Gentamycin	R	R	R	R	R	R	R	R	R	R	R	R	R	S
Clindamycin	S	R	S	R	R	S	R	R	S	R	S	S	R	S
Erythromycin	R	S	R	S	R	R	R	R	S	R	R	R	S	R
Vancomycin	S	S	S	S	S	S	S	S	S	S	S	S	S	S
Fusidic acid	R	R	S	R	R	R	S	R	R	R	R	R	S	S

Note. R — resistant strain, S — sensitive strain.

Table 3. Resistance genes in *S. epidermidis*

Resistance gene	Strain /sample type/													
	1	2	3	4	5	6	7	8	9	10	11	12	13	14
	stools	blood	tracheobronchial aspirate	oropharyngeal swab	stools	lung tissue	tracheobronchial aspirate	tracheobronchial aspirate	stools	oropharyngeal swab	blood	eye discharge	tracheobronchial aspirate	tracheobronchial aspirate
aacA-aphD	+	+	+	+	+	+	+	+	+	+	+	+	–	–
aadD	+	+	–	+	–	–	–	–	+	+	–	–	–	–
aphA	–	–	+	–	–	–	+	–	–	–	–	–	–	–
blaZ	+	+	+	+	+	+	+	+	+	+	+	+	+	+
mecA	+	+	+	+	+	+	+	+	+	+	+	+	–	+
fusB	–	+	–	+	+	+	–	–	+	+	–	–	–	–
fusC	–	–	–	–	–	–	–	–	–	–	–	–	–	–
vanA	–	–	–	–	–	–	–	–	–	–	–	–	–	–
vanB	–	–	–	–	–	–	–	–	–	–	–	–	–	–
vanZ	–	–	–	–	–	–	–	–	–	–	–	–	–	–
lnu(A)	+	–	+	–	–	–	–	+	–	–	–	–	–	+
vga(A)	–	–	–	–	–	–	–	–	–	–	–	–	–	–
vga(B)	–	–	–	–	+	–	–	–	–	–	–	–	–	–
msr(A)	–	–	–	–	–	+	+	–	–	–	+	+	–	–
mph(C)	–	–	–	–	–	–	+	–	–	–	+	+	–	–
erm(A)	–	–	–	–	+	–	–	–	–	–	–	–	–	–
erm(B)	–	–	–	–	–	–	–	–	–	–	–	–	–	–
erm(C)	+	–	+	–	–	–	+	+	–	+	–	–	–	+
vat(B)	–	–	–	–	+	–	–	–	–	–	–	–	–	–
tet(M)	–	–	–	–	–	–	–	–	–	–	–	–	–	–
tet(K)	+	–	–	–	–	–	+	+	–	–	–	–	–	+
dfr(G)	+	–	+	–	–	–	–	–	–	–	–	–	–	–
dfr(K)	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Sequence-type	ST210	ST2	ST59	ST2	ST87	ST2	ST59	ST218	ST22	ST2	ST59	ST59	ST19	ST173

Note. Symbols + and – represent the presence and absence of the resistance gene in the strain genome. Gene *aacA-aphD* mediates resistance to amikacin, gentamycin, kanamycin, and tobramycin; gene *aadD* mediates resistance to amikacin, kanamycin, neomycin and tobramycinl; *aphA* mediates resistance to kanamycin and neomycin; *blaZ* — to penicillins; *mecA* — to beta-lactam antibiotics; *fusB* and *fusC* to fusidic acid; *vanA*, *vanB*, and *vanZ* to vancamycin; *lnu(A)*, *vga(A)*, and *vga(B)* — to licosamides (clindamycin); *msr(A)* and *mph(C)* — to macrolide antibiotics (erythromycin); *erm(A)*, *erm(B)*, and *erm(C)* — to macrolide antibiotics and lincosamides; *vat(B)* — to streptogramin B; *tet(M)* and *tet(K)* — to tetracycline; *dfr(G)* and *dfr(K)* – to trimethoprim.

7, 11, and 12 harbored the *msr(A)* gene, genomes of strains 7, 11, and 12 harbored the *mph(C)* genes. Those genes mediate bacterial resistance to macrolides, including erythromycin.

Of 3 genes responsible for resistance to both macrolides and lincosamides [8, 11], 2 were carried by the studied strains. Gene *erm(C)* was present in strains 1, 3, 7, 8, 10, and 14; gene *erm(A)* was present in one strain (strain 5). Gene *erm(B)* was absent in the studied genomes.

For an adequate therapy of *S. epidermidis*-associated infections, phenotypic resistance of its strains to antibiotics must be tested. In our work we attempted to identify genes that mediate resistance to different groups of antibiotics. Of particular interest are genes that determine bacterial resistance to drugs that have not been included in treatment regimens so far. First, there is a chance of horizontal gene transfer of mobile genetic elements and plasmids between *S. epidermidis* and other bacteria. Second, the presence or absence of resistance genes in the genome of the studied strain makes it possible to assess its genetic characteristics.

The *vat(B)* gene that mediates resistance to streptogramin B and possibly lincosamides [8] was observed in only one strain (strain 5). Of 2 genes conferring tetracycline resistance, only one gene *tet(K)* was detected in the studied genomes (strains 1, 7, 8, and 14). The other gene *tet(M)* was not observed in the studied strains. Strains 1 and 3 carried gene *dfr(G)* associated with trimethoprim resistance. Another trimethoprim resistance gene *dfr(K)* was not present in the genomes.

Results of virulence-associated gene search in *S. epidermidis* are shown in Table 4.

DISCUSSION

In spite of sequence type diversity observed in the studied strains (8 variants were detected), 10 strains of 14 belonged to the major clonal complex CC2. These strains belonged to ST2, ST22, ST59 and ST87 sequence types. According to the literature, ST59 and ST22 are the most common sequence types of *S. epidermidis* circulating in the hospital environment [2, 3]. Sequence type ST2 is an progenitor source for one of 2 major clusters (cluster I) of the CC2 clonal complex. A multicenter study carried out by Miragaiet et al. in 2007 in 18 countries demonstrated worldwide dissemination of *S. epidermidis* clonal lineages in the hospital environment [3]. Similar results were obtained in Russia in 2013 [2]. This prompts a supposition that types belonging to the CC2 clonal complex have certain genetic characteristics that allow them to circulate and persist in hospitals for a long time, such as increased resistance to antibiotics or a special combination of antibiotic resistance genes in chromosomal elements, increased ability to form biofilms, etc. Further in-depth research is required aimed to analyze genetic properties of CC2 strains isolated from clinical samples.

The studied staphylococcal strains carry a varying range of genes conferring resistance to antimicrobial drugs. As confirmed by other studies [1, 6, 7, 14], different strains have different sets of resistance genes. Penicillin resistance genes were detected in all 14 strains (100 %); methicillin resistance genes were detected in 13 (93 %) strains; aminoglycoside resistance genes — in 12 strains (86 %); macrolide resistance genes – in 10 strains (71 %); lincosamide resistance genes — in 7 strains (50 %); fusidic acid resistance genes — in 6 strains (43 %); tetracycline resistance genes — in 4 strains (29 %); trimethoprim resistance genes — in 2 strains (14 %). None of the strains carried vancomycin resistance genes. Some strains carried a set of 1 (strain 13) to 8 (strains 1 and 7) antibiotic resistance genes.

It should be noted that the presence of a resistance gene does not imply its expression in the amount sufficient to form a phenotype. However, a nucleotide sequence can either directly form a resistance phenotype or serve as a source for further evolution that will ultimately yield phenotypic resistance.

On the other hand, resistance genes are not the only source of phenotypic antibiotic resistance. Resistance may be associated with modifications or inactivation of native enzymes triggered by mutations in the native genes. For example, staphylococcal resistance to methicillin may be associated not only with the presence of cassette chromosome mecs but also with modifications of the penicillin-binding protein. Besides, reduced sensitivity to antibiotics may be a result of increased activity of efflux pumps that pump antibiotics out of the cell. There are other known types of antibiotic resistance

Table 4. Genes associated with virulence in *S. epidermidis*

Strain ID	Gene						
	icaA	*icaD*	*IS256*	*aap*	*embp*	*atlE*	*aae*
1	–	–	+	–	+	+	+
2	+	+	+	+	–	+	+
3	–	–	–	+	+	+	+
4	+	+	+	+	–	+	+
5	–	–	+	–	+	+	+
6	+	+	+	+	–	+	+
7	–	–	–	+	+	+	+
8	+	+	+	+	+	+	+
9	+	+	+	–	+	+	+
10	+	+	+	+	–	+	+
11	–	–	–	+	+	+	+
12	–	–	–	+	+	+	+
13	–	–	–	+	+	+	+
14	+	+	+	+	+	+	+

Note. Symbols + and – represent the presence and absence of a resistance gene in the strain genome.

mechanisms. In this work, we did not aim to analyze nucleotide sequences or their localization, detect mutations capable of affecting gene expression or evaluate gene expression levels. The presence of resistance genes positively correlated with phenotypic resistance to antibiotics in penicillin, lincosamides, and macrolides. Phenotypic resistance to other antibiotics was demonstrated by those strains that carried resistance genes and those that did not, meaning that bacteria can employ different resistance mechanisms. Therefore, our research is going on.

To sum up, nosocomial strains isolated from patients of the Neonatal Intensive Care Unit of Kulakov Research Center for Obstetrics, Gynecology and Perinatology carry a wide range of antibiotic resistance genes.

The number of virulence-associated genes found in *S. epidermidis* strains is fairly large. However, the majority of these genes can promote both pathogenicity and commensalism. Therefore, the presence of such genes in *S. epidermidis* does not allow us to conclude whether the strain is pathogenic or harmless [1, 14, 15]. However, we did observe a correlation between the presence of virulence factors and pathogenicity of nosocomial strains [16–18].

All 14 strains carried genes *aae* and *atlE*. Both of them encode bifunctional proteins that can act as autolysins and adhesins and participate in biofilm formation. These genes were very common for the studied *S. epidermidis* strains. Other genes associated with virulence were found only in some of the studied strains. The studied strains carried genes *aap* and *embp* and genes *icaA* and *icaD* of the *ica*-operon. Gene *aap* encodes protein Aap, which is the most important factor of protein-dependent biofilm formation. Gene *embp* participates in biofilm formation, and genes of the *ica*-operon mediate synthesis of exopolysaccharide intracellular adhesins that have a role in biofilm formation and immune evasion.

Insertion sequence *IS256* was found in some strains, positively correlating with strain virulence [17].

Thus, a set of genes associated with virulence varied in different nosocomial strains isolated from patients of the Neonatal Intensive Care Unit of Kulakov Research Center for Obstetrics, Gynecology and Perinatology. Some strains of *S. epidermidis* harbored from 4 (strains 1, 3, 5, 7, 11, 12, and 13) to 7 (strains 8 and 14) genes associated with virulence. Interestingly, strains 1 and 7 carried the highest number of resistance genes and the lowest number of virulence genes.

CONCLUSIONS

We have studied genetic diversity of 14 nosocomial strains of *S. epidermidis* isolated from patients of the Neonatal Intensive Care Unit of Kulakov Research Center for Obstetrics, Gynecology and Perinatology. The strains were classified into 8 sequence types; 10 of 14 strains belonged to the major clonal complex CC2. The studied strains were found to carry a wide range of antibiotic resistance genes and virulence genes. We identified 15 different genes conferring resistance to aminoglycosides, beta-lactams, fusidic acid, macrolides, lincosamides, streptogramin B, tetracycline, and trimethoprim. We also identified genes associated with virulence (*aae*, *atlE*, *aap*, *embp*, *icaA*, *icaD*) and found that their frequency in the studied strains varied. Additionally, insertion sequence *IS256* was identified.

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POLYMORPHISM OF THE *DTXR* GENE IN THE CURRENTLY EXISTING STRAINS OF *CORYNEBACTERIUM DIPHTHERIAE*

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The pathogenic mechanism used by *Corynebacterium diphtheriae* is attributed to the ability of the diphtheria toxin to disrupt protein synthesis in human cells. Diphtheria toxin production is regulated by the DtxR protein. The latter is involved in the iron-mediated repression of the toxin gene and coordinates activities of other genes essential for the survival of *C. diphtheriae*. The DtxR-encoding gene occurs in both toxigenic and non-toxigenic strains; therefore it can be used to analyze the population structure of the species. In our work we have studied 45 strains of *C. diphtheriae* isolated in the Russian Federation in 2010–2015. These strains were analyzed to reveal that gene *dtxR* is a highly conservative region of *C. diphtheriae* genome that can be found in all members of the studied species. The majority of the discovered polymorphisms were synonymous (16 of 18 single nucleotide polymorphisms identified). In spite of the low phylogenetic signal, the allelic variant of *dtxR* was associated with the strain's phenotype (biovar, toxigenicity). The obtained data indicate the presence of aggressive negative selection aimed to maintain the existing protein sequence in the population. Based on the results, we recommend *dtxR* polymerase chain reaction as an additional technique for pathogen identification, which is especially relevant considering the increasing prevalence of the disease associated with non-toxigenic *C. diphtheriae* strains.

Keywords: diphtheria, *Corynebacterium diphtheriae*, *dtxR*, multilocus sequence typing, metalloregulatory proteins

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ПОЛИМОРФИЗМ ГЕНА *DTXR* У СОВРЕМЕННЫХ ШТАММОВ *CORYNEBACTERIUM DIPHTHERIAE*

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Считается, что патогенез *Corynebacterium diphtheriae* основан на воздействии дифтерийного токсина на синтез белка в клетках человека. Регуляция синтеза токсина находится под контролем белка DtxR. Данный белок осуществляет железоопосредованную репрессию гена дифтерийного токсина, а также координирует работу множества других генов, необходимых для нормальной жизнедеятельности *C. diphtheriae*. Ген, кодирующий DtxR, можно использовать для анализа популяционной структуры вида, так как он присутствует в геноме как токсигенных, так и нетоксигенных штаммов. В работе было изучено 45 штаммов *C. diphtheriae*, выделенных на территории Российской Федерации в 2010–2015 гг. Анализ этих штаммов показал, что ген *dtxR* обнаруживается у всех представителей вида и является высококонсервативным участком генома *C. diphtheriae*. Большинство выявленных полиморфизмов были синонимичны (16 из 18 однонуклеотидных замен). Несмотря на низкий уровень филогенетического сигнала, аллельный вариант *dtxR* был ассоциирован с биологическими признаками штамма (биовар, токсигенность). Полученные данные свидетельствуют о высокой активности отрицательного отбора, направленного на поддержание в популяции существующей последовательности белка, и позволяют рекомендовать наработку фрагментов гена *dtxR* методом полимеразной цепной реакции в качестве дополнительного метода идентификации возбудителя, что особенно актуально в условиях растущего числа заболеваний, ассоциированных с нетоксигенными штаммами *C. diphtheriae.*

Ключевые слова: дифтерия, *Corynebacterium diphtheriae*, *dtxR*, мультилокусное сиквенс-типирование, металлорегуляторные белки

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In spite of successful vaccination strategies, sporadic cases of diphtheria still occur, and the infection remains a serious health issue. Virulence of *Corynebacterium diphtheriae* is associated with its ability to produce a diphtheria toxin encoded by the *tox* gene [1]. Its pathogenic mechanism is based on ADP ribosylation of the elongation factor 2 that disrupts protein synthesis in human cells [2]. It should be noted that the presence of the *tox* gene in the *C. diphtheriae* genome does not necessarily confer toxigenicity. There are nontoxigenic tox-bearing strains (NTTB strains) that have lost their ability to synthesize a fully functional toxin following a series of mutations [3, 4].

Although *tox* is a part of the phage genome, iron-mediated regulation of toxin expression is exerted by the iron-sensing regulator DtxR, the product of the chromosomal gene *dtxR*. Thus, *tox* transcription directly depends on iron homeostasis, as low iron levels trigger *tox* expression followed by synthesis of the diphtheria toxin [5].

The *dtxR* gene is present in both toxigenic and nontoxigenic strains [6] meaning that it has functions other than regulation of diphtheria toxin synthesis. The DtxR regulon is reported to contain 20 more loci, including genes responsible for iron metabolism. To date, the DtxR protein of *C. diphtheriae* is known to regulate siderophore synthesis, a high-affinity transport system (ciuABCDEFG) and transcription of 3 loci involved in heme-monooxygenase (*hmuO*) activity [1, 7]. DtxR may also have a role in regulating bacterial virulence [7].

Microorganisms need large amounts of iron which is not so easy to acquire. However, iron excess stimulates production of toxic reactive oxygen species. Mammals have developed a mechanism of nonspecific defense against infections that relies on reducing the levels of unbound iron by specific iron-binding proteins [8, 9].

Therefore, survival and dissemination of the pathogen in the host depends on its ability to acquire different metal ions from protein complexes. For that, the pathogen employs various uptake mechanisms. Gene expression is controlled by metalloregulatory proteins - highly conserved transcriptional regulators [10, 11]. Once they bind to a specific metal ion, these regulators change their conformation and trigger or repress binding of the active site to the gene operator [12].

DtxR is a typical example of a metalloregulatory protein. Crystallography demonstrates that in its inactive state DtxR is a monomer that consists of two domains. A large conserved N-terminal domain contains two binding sites for iron ions and a helix-turn-helix motif that can bind to DNA; a smaller, less conserved C-terminal domain resembles the SH3 domain of eukaryotes. Ferrous ions bind to the binding sites rendering the repressor active. Once it is activated, dimerization occurs [13].

The two DtxR domains are linked by a proline-rich peptide segment. When the repressor is inactive, this segment binds to the SH3-resembling domain resulting in the formation of a prolylpeptide-SH3 complex (Pr-SH3) and stabilizing the repressor in its inactive state. After ferrous ions bind to the N-terminal domain triggering DtxR activation, the Pr-SH3 complex dissociates and the proline segment stabilizes helical segments of the N-terminal domain, which leads to dimerization of two protein subunits [14].

Considering the role of DtxR in *C. diphtheriae* survival, the *dtxR* gene must be studied to evaluate the pathogenic potential of *C. diphtheriae*, elucidate dynamics of circulating strains and assess feasibility of *dtxR* as a target for the PCR-based diagnosis of diphtheria or other infections associated with nontoxigenic *C. diphtheria* strains. The aim of this work was to identify genetic polymorphisms of DtxR and to analyze the population structure of *C. diphtheriae* strains circulating in Russia.

METHODS

We studied genotypic characteristics of 45 strains of *C. diphtheriae* (bv. gravis and bv. mitis) isolated in 2010–2015. The study was conducted at the Reference center for Measles, Parotitis, Rubella, Pertussis, and Diphtheria of Gabrichevsky Moscow Research Institute of Epidemiology and Microbiology. *C. diphtheriae* strains were obtained from bacterial laboratories of the institutions for disease prevention and centers for hygiene and epidemiology located in 14 different regions of Russia, where the strains had been isolated for diagnostic or preventive screening or for the purpose of epidemiological research. The following collection strains were used: *C. diphtheriae* (State Research Center for Applied Microbiology & Biotechnology, Obolensk, Russia) and *C. diphtheriae* PW 8 (Therapeutic Products Regulatory Research Center, Moscow, Russia). Besides, the following strains were used as PCR negative control, 1 strain per species: *C. ulcerans*, *C. pseudotuberculosis*, *C. amycolatum*, *C. glucuronolyticum*, *C. xerosis*, *C. afermentans subsp. afermentans*, *C. afermentans subsp. lipophiium*, *C. coyleae*, *C. pseudodiphtheriticum*, *C. macifaciens*, *C. simulans*, and *C. durum* from the collection of Gabrichevsky Research Institute.

The strains were isolated following the guidelines of the *Laboratory Diagnosis of Diphtheria* manual (Guidelines 4.2.698–98 and 4.2.3065–13). The isolates were seeded onto the solid tellurite blood agar base containing 2 % agar (Microgen, Russia), 10 % bovine blood (LeiTran, Russia), and 0.02 % potassium tellurite (State Research Center for Applied Microbiology & Biotechnology, Russia). Then the cultures were thermostated for 24–48 h at 37 °C. Morphological, toxigenic and biochemical profiles of the grown cultures were prepared according to Guidelines 4.2.698-98 and 4.2.3065–13 mentioned above using the biochemical test system DS-DIPH-CORYNE (Diagnostic Systems, Russia).

Chromosomal DNA was extracted by boiling from a freshly grown 24-hour old *C. diphtheriae* culture. A culture sample was picked up with a sterile loop and suspended in 100 μl of deionized water, incubated for 20 min at 95 °C and centrifuged. The supernatant was used for the PCR assay.

PCR amplification of the *dtxR* gene of *C. diphtheriae* was performed using a pair of primers to cover the entire region of the studied gene: a previously proposed F1 5'-GGGACTACAACGCAACAAGAA-3' [15] and R1 5'-TCATCTAATTTCGCCGCCTTTA-3' designed by the PerlPrimer application, v1.1.21 [16]. Specificity of primers was checked using BLASTn by comparing their sequences to similar sequences of other *Corynebacterium* species obtained from the NCBI Nucleotide database.

The reaction mix contained a PCR buffer with 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 μM of each primer, 200 μM of each dNTP, 1 μL of the DNA solution, and 1 unit/50 μL Taq DNA polymerase (Fermentas, Lithuania). Amplification was performed in the Tertsik amplifier (DNA Technology, Russia) operated in the automatic mode The amplified fragments were analyzed by 1.5 % agarose gel electrophoresis. Sequencing of the obtained fragments was performed by Evrogen, Russia.

C. diphtheriae strains were genotyped by multilocus sequence typing (MLST) according to the international protocol [17] using fragments of sequences of 7 housekeeping genes, namely *atpA* (encodes the α-subunit of ATP synthase),

dnaE (encodes the α-subunit of the DNA polymerase III holoenzyme), *dnaK* (encodes the Hsp70 chaperone), *fusA* (encodes elongation factor G), *leuA* (encodes 2-isopropylmalate synthase), *odhA* (encodes components E1 and E2 of the 2-oxoglutarate dehydrogenase complex), and *rpoB* (encodes the β-subunit of RNA-polymerase). Allelic profiles were identified for each strain.

The obtained sequences were compared to the nucleotide sequences published in GenBank. The sequence of the *dtxR* gene of *C. diphtheriae* PW8 was used as a reference (Genbank NC_016789.1).

Nucleotide sequences were aligned using the MUSCLE algorithm [18]. Polymorphisms were mapped to the protein structure based on the PDB data. Queries to the NCBI Nucleotide database were run using BLASTn. To identify alleles of the housekeeping genes, the PubMLST software was used. To estimate selection pressure on the *dtxR* gene, we applied the Nei-Gojobori method and calculated the K_a/K_s ratio, where K_a is the number of nonsynonymous substitutions per site and K_s is the number of synonymous substitutions per site [19]. Fisher's exact test was performed on contingency tables using the AS159 algorithm [20] and R 3.3.2. Phylogenetic trees were reconstructed by neighbor-joining based on the comparison of *dtxR* nucleotide sequences and included sequences of *C. diphtheriae* PW8, *C. diphtheriae* NCTC 13129 and *C. diphtheriae* 178-01 (Genbank NC_016789.1, BX248353.1 and NZ_JZUJ01000001.1). Evolutionary distances were computed using the Maximum Composite Likelihood method [21] and scaled as units of substitutions per site. Evolutionary analysis was performed by MEGA7 v.7.0.21 [22]

RESULTS

PCR amplification revealed the presence of the *dtxR* gene in all studied toxigenic and nontoxigenic strains of *C. diphtheriae*. Besides, PCR results came out negative for all allied species.

The samples of 45 *C. diphtheriae* strains were sequenced and the obtained sequences were compared to the *dtxR* sequences retrieved from GenBank revealing polymorphisms at 18 positions: 66, 126, 225, 273, 358, 402, 440, 474, 504,507, 516, 558, 564, 579, 639, 640, 654 and 685 (Table 1).

The majority of substitutions relative to the reference sequence were synonymous. Specifically, the most frequent single nucleotide substitution at position 273 of the *dtxR* gene did not affect the protein sequence. This substitution was observed in 14 strains.

Polymorphisms at positions 440 and 640 that resulted in A147V and L2141substitutions, respectively, seemed to have no significant effect on the DtxR function. According to the 3D protein structure published in PDB (ID 2QQ9), amino acid at position 147 is found in the unstructured proline-rich (Pr) region. Since this segment participates in protein dimerization, we cannot rule out a possible effect of the amino acid substitution on DtxR activation. It appears that substitution of leucine for isoleucine at position 214 of the C-terminal domain does not have any effect on protein folding because these amino acids have similar properties. Thus, nonsynonymous substitutions are very likely to produce no effect on the DtxR function.

Many of the identified sequence variants are well known and were described previously by other researchers. However, we were able to identify a new single nucleotide polymorphism at position 358 that also has no effect on the amino acid sequence. A nucleotide query to the NCBI Nucleotide database performed by BLASTn returned no results.

We observed various combinations of polymorphisms in the *dtxR* gene relative to the reference sequence. Based on the discovered combinations of nucleotide sequences, *C. diphtheriae* isolates were distributed into several groups (Table 2).

More than a half (55 %) of identified nucleotide sequences differed from the reference sequence. It should be noted that the new substitution at position 358 was observed in one strain only (group 5) that had the least number of substitutions compared to the reference sequence, including nonsynonymous polymorphisms.

Based on the alignment of *dtxR* sequences, we constructed a phylogenetic tree (see the Figure).

Table 1. Frequency of nucleotide substitutions in the *dtxR* gene of the studied strains of *C. diphtheriae* relative to the strain PW8

Position of nucleotide in the *dtxR* gene	Nucleotide substitution	Encoded amino acid	Amino acid substitution	Number of strains
66	A–T	22	–	5
126	C–T	42	–	5
225	T–C	75	–	6
273	C–T	91	–	14
358	T–C	120	–	1
402	T–A	134	–	1
440	C–T	147	Alanine (A) – Valine (V)	6
474	C–T	158	–	6
504	T–A	168	–	6
507	C–T	169	–	6
516	T–C	172	–	6
558	C–T	186	–	2
564	T–A	188	–	6
579	C–T	193	–	2
639	C–T	213	–	4
640	C–A	214	Leucine (L) – Isoleucine (I)	2
654	T–C	218	–	2
685	C–T	229	–	1

Table 2. Combinations of polymorphisms of the dtxR gene in *C. diphtheriae* strains

Group of strains	Number of strains (n = 45)	Position of nucleotide in *dtxR* nucleotide sequence																	
		66	126	225	273	358	402	440	474	504	507	516	558	564	579	639	640	654	685
1	20 (44 %)	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
2	5 (11 %)	*	T	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
3	9 (20 %)	*	*	*	T	*	*	*	*	*	*	*	*	*	*	*	*	*	*
4	5 (11 %)	*	*	C	T	*	*	*	*	*	*	*	*	*	*	T	*	*	*
5	1 (2 %)	*	*	C	*	C	A	**T**	T	A	T	C	T	A	T	T	**A**	C	T
6	1 (2 %)	T	*	*	*	*	*	**T**	T	A	T	C	T	A	T	T	**A**	C	*
7	4 (9 %)	T	*	*	*	*	*	**T**	T	A	T	C	*	A	*	*	*	*	*
Reference strain PW8	–	A	C	T	C	T	T	C	C	T	C	T	C	T	C	C	C	T	C

Note. * — a match with the reference; **T**, **A** — polymorphism with amino acid substitution.

The branching order is an approximation to some extent, due to the similarity of the analyzed sequences and hence a weak phylogenetic signal. The tree in the dendrogram is not rooted due to the lack of possibility to select an appropriate outgroup.

We also analyzed correlations between group composition and toxigenicity, biovars and sequence types (ST) determined by MLST (Table 3). Profiles of NTTB strains were previously described in [23].

Based on the distribution of toxigenic and nontoxigenic strains of different biovars with regard to the allelic variants of *dtxR*, Fisher's exact test was performed. The test was performed on 2 × 7 contingency tables to examine the association between a biovar type and the allelic variant of *dtxR* ($p = 0.00078$) and on 3 × 7 contingency tables to examine the association between toxigenicity and the allelic variant of *dtxR* ($p = 2.8 \cdot 10^{-9}$). The obtained results prompt us to conclude that the associations between a biovar type/toxigenicity and the allelic variant of *dtxR* are not accidental and implicate a phylogenetic signal — a sum of associations between the allelic variant of the gene and biological characteristics of the strains. Distribution of sequence types was nonuniform, identical sequence types were rarely found in one group.

To assess selection pressure on the *dtxR* gene, we calculated the K_a/K_s ratio (0.0526). The obtained value ($K_a/K_s < 1$) indicates a strong negative selection, i.e., selection pressure is aimed at maintaining the current protein sequence [24].

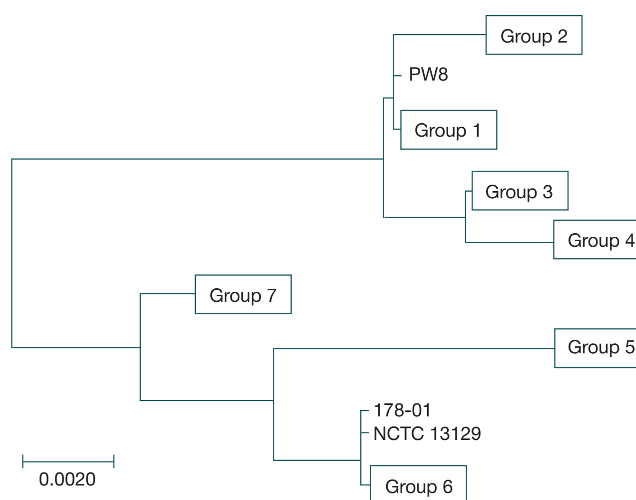
DISCUSSION

Our study confirmed the essential role of DtxR in the viability of toxigenic and nontoxigenic strains of *C. diphtheriae*. The observed polymorphisms provide new information of the variability of its strains in Russia. *dtxR*–related nucleotide and amino-acid substitutions were studied previously under various conditions with regard to diphtheria dissemination [25–27]. This work was conducted against the background of sporadic incidence. We performed a comprehensive analysis of nucleotide sequences and assessed their correlation with strain toxigenicity, biovars and sequence types. This approach allowed us to better understand the structure of the population of currently circulating *C. diphtheriae* strains.

The hypothesis about the significant effect of horizontal gene transfer on the structure of *C. diphtheriae* population was discussed earlier [17]. There are works describing transfer mechanisms for genes conferring antibiotic resistance [28] and virulence [29]. Components of the DtxR regulon may vary in different strains of *C. diphtheriae* due to the loss, acquisition, or partial deletion of genes responsible for iron provision and hence *tox* expression [1, 30]. The discovered correlation between a sequence type and the allelic variant of *dtxR* proves the idea that homologous recombination in *C. diphtheriae* does not completely block the phylogenetic signal [17]. At the same time, the analysis of the population structure did not reveal any direct correlation between a biovar and the *dtxR* allele meaning that there is no phylogenetic basis for such classification, which is mainly determined by the frequency of horizontal gene transfer [31].

Less than a half (45 %) of the studied sequences were found to be identical to the reference sequence. Of 13 polymorphisms, only 1 was identified as new (position 358), but it did not affect the amino acid sequence. The most frequent was the synonymous polymorphism at position 273 observed in 14 strains. Our work demonstrates that Russian strains carry a smaller range of variants of the primary DtxR structure [26, 27].

It should be noted that although *dtxR* affects bacterial resistance to oxidative stress, under normal conditions it is not a critical gene for *C. diphtheria* [32], as was proved in the experiment with the mutant DtxR-defective strain [33]. However, we did not observe any significant changes in the studied sequences that could result in the synthesis of



The phylogenetic tree of *C. diphtheriae* strains based on the *dtxR* gene sequences

The tree is scaled to 0.1 substitutions per 200 b. p.; branch lengths correspond to the evolutionary distances used to construct the tree. Sequences within groups are identical.

Table 3. Group composition according to polymorphism combinations in the *dtxR* gene of *C. diphtheriae* strains

Group	Number of substitutions in *dtxR* relative to strain PW8	Number of strains	Biovar (number of strains)	Toxigenicity (number of strains)	Sequence type
1	–	20	mitis (18), gravis (2)	notoxigenic (15)	*
				NTTB strains (3)	76
				toxigenic (2)	5, 46
2	1	5	mitis	NTTB strains	40
3	1	9	gravis (1), mitis (8)	toxigenic (8)	25
				notoxigenic (1)	123
4	3	5	mitis	toxigenic	28, 67
5	15	1	mitis	notoxigenic	–
6	12	1	gravis	toxigenic	8
7	7	4	gravis	toxigenic	8

Note. * — MLST non performed, – — ST not found in the database.

a functionally inactive protein. There were two nucleotide polymorphisms that did result in amino acid substitutions, but the analysis of PDB data showed that those substitutions did not affect protein folding. Perhaps, functionally important polymorphisms of the *dtxR* gene may impair strain adaptation to the mammalian host limiting distribution of alleles that lead to the synthesis of the defective protein among the *C. diphtheriae* population. The value of the K_a/K_s ratio proved that DtxR is controlled by the stabilizing selection.

The pathogenic potential of *C. diphtheriae* does not always depend on strain's ability to produce the diphtheria toxin. For example, nontoxigenic strains of *C. diphtheriae* are becoming an increasing source of severe infection causing endocarditis [34], arthritis [35] and osteomyelitis [36]. Nontoxigenic strains are especially dangerous for patients with compromised immunity [37, 38]. This necessitates a more comprehensive approach to *C. diphtheriae* identification as currently existing methods are aimed at detecting toxigenic strains only.

Our findings confirmed the presence of the *dtxR* gene in both toxigenic and nontoxigenic strains of *C. diphtheriae* proving the feasibility of PCR-based identification proposed earlier [39]. Sequencing demonstrated that polymorphisms occurred mainly in the C-terminal domain of DtxR [15]. However, nucleotide substitutions were also observed in other gene regions (positions 66, 126, 225, 273, and 358). One of such substitutions (position 126) was found in 5 strains (group 2) in the region corresponding to the primer that had been proposed earlier for PCR- *dtxR* [15, 39]. The pair of primers used in this study proved their high specificity confirmed by zero false-positive results. This, PCR-based identification of *C. diphtheriae* is a promising technique for the diagnosis of diphtheria and infections associated with nontoxigenic strains.

CONCLUSIONS

We analyzed the structure of *C. diphtheriae* population based on the allelic variants of the *dtxR* gene. The analysis revealed that 55 % of strains had sequences different from the reference sequence. The majority of the discovered polymorphisms were synonymous. The absence of wild strains with defective DtxR and a high similarity of the analyzed nucleotide sequences indicate a strong negative selection aimed to maintain the currently existing repressor sequence.

Homologous recombination attenuates the phylogenetic signal but does not block it completely. We discovered associations between the allelic variants of *dtxR* and toxigenicity/biovar type. The obtained results allow us to conclude that *dtxR* represents a conserved sequence. We recommend PCR-*dtxR* as an accurate method for identification of toxigenic and nontoxigenic strains of *C. diphtheriae*.

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AMINOPYRIDINE- AND AMINOPYRIMIDINE-BASED SERINE/THREONINE PROTEIN KINASE INHIBITORS ARE DRUG CANDIDATES FOR TREATING DRUG-RESISTANT TUBERCULOSIS

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Tuberculosis (TB) is the world's deadliest bacterial infection. Its causative agent *Mycobacterium tuberculosis* evolves into rapidly spreading multidrug-resistant and extensively drug-resistant (MDR and XDR) strains, which complicates the treatment. Therefore, the use of novel target-specific chemical compounds is crucial for the development of effective antituberculosis agents. Serine/threonine protein kinases (STPKs) of *M. tuberculosis* are currently considered as attractive drug targets. In turn, aminopyridines and aminopyrimidines that have not been used for TB treatment so far exhibit inhibitory activity towards STPKs. In this study we screened 192 aminopyridine- and aminopyrimidine-based compounds using the M*ycobacterium smegmatis aphVIII*+ test system designed to screen for active STPKs inhibitors. First, we selected 53 compounds with subinhibiting concentrations of up to 100 nmol/disk. Of them, 22 showed STPKs-inhibiting activity in the test system, which was confirmed *in vitro* on the *M. tuberculosis* PknA protein with a maximum of 26.9 ± 6.1 %. Toxicity testing was performed *in vitro* on human embryo fibroblasts using the MTT-assay. Ultimately, 3 relatively active and relatively non-toxic STPKs inhibitors were selected for further research as drug candidates for MDR-TB treatment.

Keywords: tuberculosis, multidrug resistance, serine/threonine protein kinases, aminopyridines, aminopyrimidines, inhibitors, *Mycobacterium tuberculosis*, *Mycobacterium smegmatis*, *aphVIII*

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ИНГИБИТОРЫ СЕРИН-ТРЕОНИНОВЫХ ПРОТЕИНКИНАЗ КЛАССОВ АМИНОПИРИДИНОВ И АМИНОПИРИМИДИНОВ — КАНДИДАТЫ В ПРЕПАРАТЫ ДЛЯ ЛЕЧЕНИЯ ЛЕКАРСТВЕННО-УСТОЙЧИВЫХ ФОРМ ТУБЕРКУЛЕЗА

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Туберкулез — самая смертоносная бактериальная инфекция из известных человеку, при этом ее лечение осложнено появлением и быстрым распространением штаммов возбудителя, *Mycobacterium tuberculosis*, с множественной и широкой лекарственной устойчивостью (МЛУ и ШЛУ). В результате главным требованием к разрабатываемым противотуберкулезным препаратам является использование новых классов химических соединений, поражающих новые биомишени. Серин-треониновые протеинкиназы (СТПК) — перспективные мишени, а аминопиридины и аминопиримидины, ранее не применявшиеся в качестве противотуберкулезных препаратов, имеют предсказанную активность в отношении СТПК. В данной работе в тест-системе *Mycobacterium smegmatis aphVIII*+, предназначенной для отбора ингибиторов СТПК на клеточном уровне, был проведен скрининг 192 соединений двух указанных классов. Сначала отобрали 53 соединения с субингибирующей концентрацией до 100 нмоль/диск. Из них 22 соединения проявили активность в тест-системе как ингибиторы СТПК, которая была подтверждена *in vitro* на белке PknA *M. tuberculosis* (наивысшее значение показателя ингибирования — 26,9 ± 6,1 %). Также отобранные соединения тестировали на токсичность *in vitro* на клетках фибробластов эмбриона человека с использованием МТТ-теста. В результате для дальнейших исследований в качестве новых препаратов для борьбы с МЛУ-туберкулезом были отобраны 3 ингибитора СТПК с относительно высокой активностью и относительно низкой токсичностью.

Ключевые слова: туберкулез, множественная лекарственная устойчивость, серин-треониновые протеинкиназы, аминопиридины, аминопиримидины, ингибиторы, *Mycobacterium tuberculosis*, *Mycobacterium smegmatis*, *aphVIII*

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At present, tuberculosis (TB) is one of the deadliest infections accounting for 1.4 million deaths and 10.4 incident cases every year [1]. Its treatment is seriously complicated by increasingly emerging multidrug-resistant (MDR) strains of its causative agent *Mycobacterium tuberculosis*, i. e. strains resistant to the most effective and lowly toxic first-line drugs rifampicin and isoniazid [2]. This necessitates the use of more expensive and toxic second-line drugs [3]. A particular case of MDR is extensive drug-resistance (XDR), i. e. MDR aggravated by resistance to at least one of fluoroquinolone antibiotics and any of the second-line injectables (amikacin, kanamycin or capreomycin) [3]. Recently, there have been reports in Iran, India and South Africa of *M. tuberculosis* strains resistant to all known first- and second-line drugs (referred to as totally drug-resistant forms) [4–6]. Russia and former Soviet republics are among countries with the highest MDR-TB burden [7]: MDR is observed in one in every five incident cases, and every second patient with previously treated TB has MDR [1].

The era of antibiotics, i.e. the last 50 or 60 year, is marked directional selection of drug-resistant *M. tuberculosis* strains. Given that normal rate of random mutations is 10^{-6}–10^{-8} per cell division, it should be highly improbable for MDR to develop, considering the use of combination therapy. However, there are a number of factors that contribute to MDR development: monotherapy resulted from poor drug supply, inadequate or ineffective chemotherapy regimens, and patient's non-compliance with the treatment [8]. Repeated and prolonged use of the same drugs also has its impact: bedaquiline is the first new anti-TB drug introduced in clinical routine over the past 40 years [9].

Advances in TB treatment depend on the discovery of new drugs capable of affect new biotargets to circumvent current resistance mechanisms. Serine/threonine protein kinases (STPKs) are attractive targets as they are universal regulators of the cell cycle in pro- and eukaryotes. In particular, these enzymes regulate growth, cell division, virulence, persistence, and intrinsic antibiotic resistance in mycobacteria [10–14]. An ability to selectively inhibit STPKs has been shown for aminopyridine- and aminopyrimidine-based compounds [15]; they have not been used for TB treatment before, therefore there are no mutant strains with resistance to these compounds.

Previously we have developed and validated a *Mycobacterium smegmatis aphVIII+* test system aimed to screen for active inhibitors of mycobacterial STPKs, in particular PknA of *M. tuberculosis* [16]. This work aimed to select active STPK inhibitors from a number of aminopyridine- and aminopyrimidine-based compounds as potential drugs candidates for MDR and XDR-TB treatment.

This article was crafted from a PhD dissertation in biology defended by one of the authors in December, 2016 [17]. The results presented in the dissertation have not been previously published elsewhere, and the authors believe they are quite important and should be shared with the scientific community.

METHODS

Bacterial strains and growth conditions

Strains used in this work were *Escherichia coli* BL21 (DE3) pLysS and *M. smegmatis mc²* 155 *E. coli* were cultured in LB media (Amresco, USA); *M. smegmatis* were cultured in Lemco-Tw media (5 g/l Lemco Powder, 5 g/l NaCl, 5 g/l bacto peptone, 0.05 % Tween-80). Cultures in liquid media were incubated in the Multitron incubator shaker (Infors HT, Switzerland) at 37 °C and 250 rpm. Solid culture media contained 2.0 % agar. The culture medium used in the test system was M290 Soyabean Casein Digest Agar by HiMedia, India.

Protocol for testing compounds in M. smegmatis aphVIII+ test system

M. smegmatis aphVIII+ culture was diluted 1 : 9 : 10 (culture : water : M290 medium) and seeded over the base agar layer on Petri dishes. The culture medium was supplemented with 50 µg/ml of hygromycin and 10 ng/ml tetracycline. The dishes were allowed to dry; then paper discs were placed inside the dishes, impregnated with either a studied compound or kanamycin or a combination of both. The dishes were then incubated at 37 °C until the bacterial lawn was formed. Then growth inhibition halos were measured. All experiments were conducted in 3 to 5 replicates [16].

Purification of M. tuberculosis PknA and AphVIII proteins

Synthesis of the *M. tuberculosis pknA* gene was performed by Evrogen (Russia). For more efficient expression in *E. coli*, the gene was codon-optimized. *pknA* was synthesized and cloned into pET-32a expressing vector. Plasmid DNAs containing *pknA* and *aphVIII* (pET16b-aphVIII) genes [18] were transformed into the *E. coli* BL21 (DE3) pLysS strain by calcium chloride–mediated transformation [19]. Overnight expression of the gene was induced by 1mM isopropyl-β-D-thiogalactopyranoside (IPTG; Anatrace, USA). Proteins were purified using the Ni-NTA Fast Start Kit (Qiagen, USA).

In vitro kinase assay

Inhibiting activity of aminopyridine- and aminopyrimidine-based compounds against PknA and AphVIII was assessed by the kinase reaction using Kinase-Glo Plus Luminiscent Kinase Assay Kit (Promega, USA) and the Biomek 3000 workstation (Beckman Coulter, USA); the technique applied was previously described by Baki et al. [20]. Substrate-level phosphorylation was estimated indirectly by measuring luminescence of residual ATP. Oligopeptide IVDAELTGEIPII was used as a PknA substrate, and kanamycin was used as an AphVIII substrate. Reaction was performed overnight in the working solution containing 15 mM HEPES (pH 7.4), 20 mM NaCl, 10 mM $MgCl_2$, 0.5 mM EDTA, 0.02 % Tween-20, and 0.1 mg/ml BSA.

The reaction mix (45 µl) for PknA kinase assay contained 3 µg protein, 5 µM ATP, and 50 µg substrate.

The reaction mix (45 µl) for AphVIII kinase assay contained 50 ng protein, 10 µM ATP, and 5 µg substrate.

Cytotoxicity of compounds

Cytotoxicity of aminopyridine- and aminopyrimidine-based compounds was estimated by the MTT assay using human embryonic fibroblasts derived from fetal skin and muscles (HEF-4). Cell viability was estimated by color development accompanying tetrazole reduction to formazon by mitochondrial dehydrogenases. Measurements were recorded on the Beckman Coulter DTX 880 Multimode Detector (a plate reader by Beckman Coulter, USA) at excitation wavelength of 595 nm. Absorbance from the wells of cells cultured with the control media was taken as 100 % [21].

RESULTS

Screening for active STPK inhibitors in the M. smegmatis aphVIII+ test system

Using the disc method, we selected a number of aminopyridine- and aminopyrimidine-based compounds that exhibited STPK-inhibiting activity in the *M. smegmatis aphVIII+* test system validated earlier.

The test system employs the following principle: STPK MSMEG_5513 phosphorylates APHVIII protein in *M. smegmatis* cells enhancing their resistance to kanamycin. After an MSMEG_5513 inhibitor is added to the system, APHVIII phosphorylation decreases and the activity of the enzyme diminishes reducing resistance of bacterial cells to kanamycin. The intrinsic antimicrobial activity of the inhibitor is determined by its ability to inhibit another STPK: MSMEG_0030 (ortholog of *M. tuberculosis* PknA), a vitally important protein for mycobacteria, and possibly some other targets. In our experiment reduced resistance to kanamycin was expressed as a larger growth inhibition halo around the disc impregnated with a combination of kanamycin and an active STPK inhibitor, compared to the halo around the disk treated with kanamycin only (Fig. 1) [16].

One of the selection criteria for compounds in the *M. smegmatis aphVIII+* test system was their subinhibiting concentration. For 53 compounds it was as low as 100 nmol/disc or less, while the rest of the compounds did not have any antibacterial effect on *M. smegmatis* at this concentration. The selected compounds were tested in the test system. STPK inhibitor LCTA-1389 (11b) [22, 23] was used as a positive control. BisV, the inactive analog of standard STPK inhibitors from the indolyl maleimide family [24], was used as a negative control.

For 22 studied compounds, in solid media a bacterial growth inhibition halo around the discs treated with both kanamycin and an STPK inhibitor was significantly larger than around the discs treated with kanamycin only. The following "hit" compounds were selected for further testing of their potential as mycobacterial STPKs inhibitors (Fig. 2): 1f8, 1g8, 1e11, 1g11, 1h11, 1a12, 1c8, 2f4, 2c3, 2c6, 2a3, 2a4, 2a7, 2h11, 2h12, 2d3, 2d11, 2b4, 2b5, 2e12, 2g12, 2h12.

Inhibiting activity of selected compounds against M. tuberculosis PknA protein in vitro

The above listed compounds were tested for their ability to inhibit *M. tuberculosis* STPK PknA *in vitro* at concentrations of 200 μM (inhibitor : target molar ratio of 154 : 1). LCTA-1389

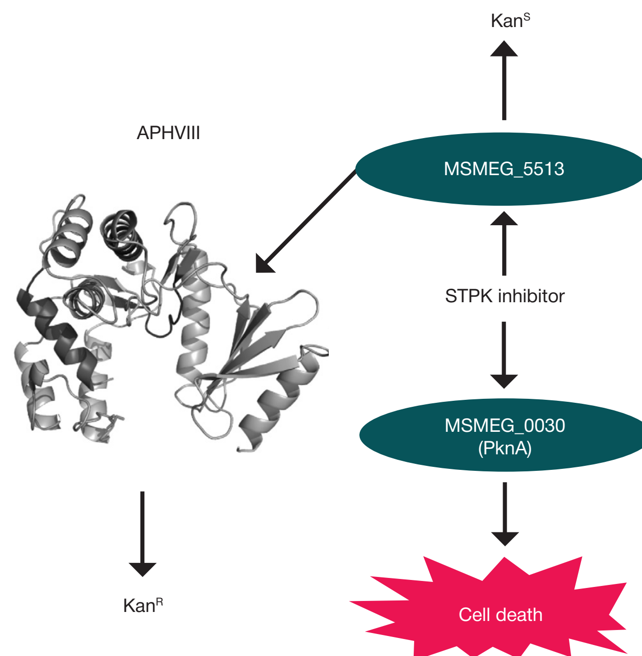

Fig. 1. Principle of *Mycobacterium smegmatis aphVIII+* test system

Kan^R — increased resistance to kanamycin, Kan^S — reduced resistance to kanamycin, STPK — serine/threonine protein kinase. Detailed description is provided in the article.

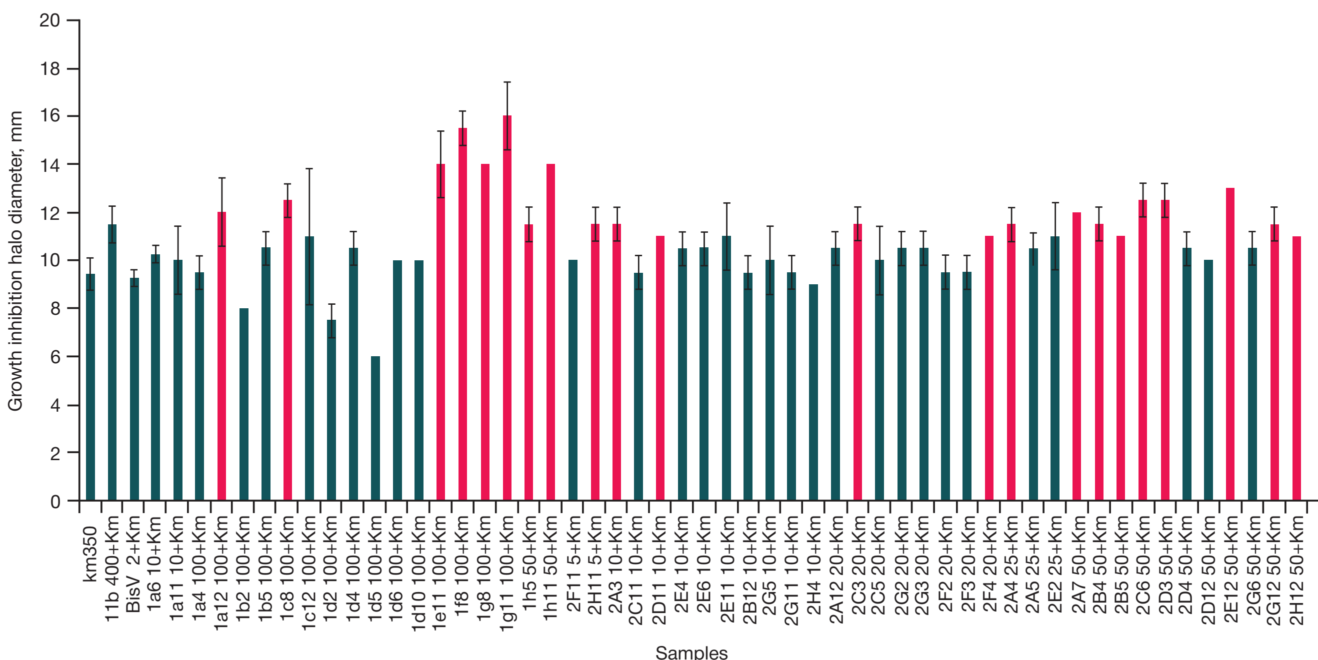

Fig. 2. Diameter of bacterial growth inhibition halos around discs impregnated with aminopyridine- and aminopyrimidine-based compounds

The compounds were tested in the *Mycobacterium smegmatis aphVIII+* test system at subinhibitory concentrations (nmol/disc; specified near the sample name) that do not produce a bacterial growth inhibition halo. Error bars represent standard deviation. Selected compounds are shown in red.

(11b) and BisV were used as positive and negative controls, respectively. Results are shown in Fig. 3. All compounds exhibited inhibiting activity similar to that of a positive control or higher, as in the case of two compounds: 1H11 (26.9 ± 6.1 %) and 2G12 (23.2 ± 2.0 %).

All of these compounds were tested at the same molar ratio (inhibitor : target) for their ability to inhibit phosphotransferase activity of APHVIII *in vitro* to make sure their activity in *M. smegmatis aphVIII*+ system was selective. Results were negative in all cases, i. e. activity of the compounds in the test system was determined by their ability to inhibit STPKs of *M. smegmatis* only and not the APHVIII protein.

Cytotoxicity of selected compounds

Cytotoxicity of the selected compounds was tested on HEF-4 cells. Based on the results, the compounds were divided into three groups: highly toxic (< 10 μg/ml; 1F8, 1G11, 2D11, 2F4, 2C3, 2A3, 2H11); moderately toxic (10–50 μg/ml; 1E11, 1G8, 1H11, 2D3, 2E12, 2G12, 2A4, 2A7); and low toxic (> 50 μg/ml; 2C6).

DISCUSSION

In the age of emerging antibiotic resistance of *M. tuberculosis* strains, effective drugs for TB treatment must meet two basic requirements: a novel mechanism of action and low toxicity.

In the past 15 years, development of antibacterial drugs, including antimycobacterial agents, was based on the biochemical targeted selection of compounds inhibiting essential bacterial enzymes. This concept had certain limitations when applied to *M. tuberculosis*: the overwhelming number of the compounds that were supposed to have anti-TB potential did not have any effect on the bacterial cell for a number of reasons, such as poor permeability of the bacterial cell wall [25]. So researchers went back to using a rapidly growing *M. smegmatis* in screening tests because its cellular wall is similar to that of *M. tuberculosis* in terms of permeability. This is how bedaquiline was discovered [26]. However, this approach dictates a need for further validation of a drug target [27].

The *M. smegmatis aphVIII*+ test system was designed to select candidate compounds based on their antimycobacterial effect and target specificity [16]. The latter was confirmed *in vitro* for the compounds selected in our experiment. However, a limitation of our study is the lack of possibility to determine a half maximal inhibitory concentration IC_{50}, which might be related to the low activity of the purified thioredoxin fusion PknA protein. A large amount of protein in the reaction mix dictated a need for the analysis of maximal soluble concentrations of the tested compounds. However, the inhibiting activity of the selected compounds that was similar or even superior (1H11 and 2G12) to that of the positive control [22, 23] and a previously demonstrated inhibiting activity of aminopyridine- and aminopyrimidine-based compounds against STPKs [15] allow us to hypothesize that these compounds may be used as effective STPK inhibitors.

Based on screening results, we have selected three compounds as potential drug candidates that exhibited the highest activity in the *M. smegmatis aphVIII*+ test system, on the PknA protein *in vitro*, and the lowest toxicity against human cell culture (Fig. 4). The most active compounds 1H11 and 2G12 were classified as moderately toxic. However, it should be noted that our screening was only the first step in the selection of "hits", which could be further optimized to enhance their effect or reduce toxicity. Their activity must be tested on *M. tuberculosis*; acute and chronic toxicity must be assessed *in vivo*. These compounds must also be tested on human STPKs to make sure their activity is selective.

CONCLUSIONS

We have demonstrated feasibility of developing a new aminopyridine- and aminopyrimidine-based drugs for MDR and XDR TB treatment. Further tests of the selected "hits" (1E11, 2C6, 2G12) are required to assess and optimize their antimycobacterial activity against *M. tuberculosis* and reduce toxicity.

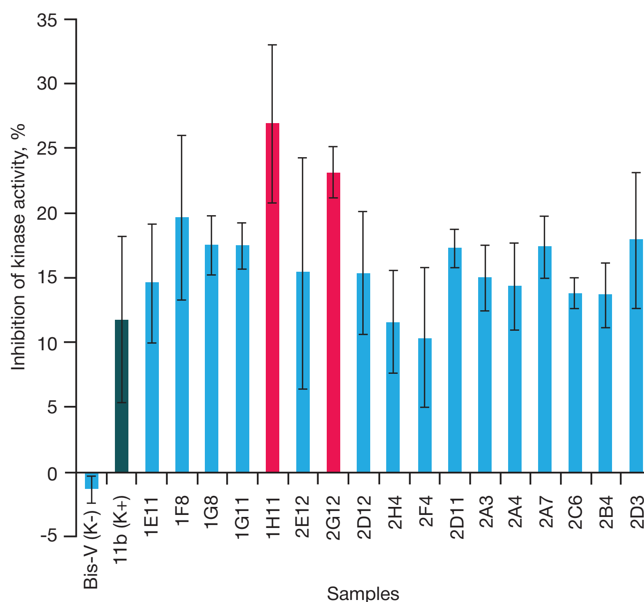

Fig. 3. Inhibition of phosphorylating activity of *Mycobacterium tuberculosis* PknA *in vitro* by compounds selected by the *Mycobacterium smegmatis aphVIII*+ test system

Error bars represent standard deviation. Positive control is shown in green. The most active compounds are shown in red.

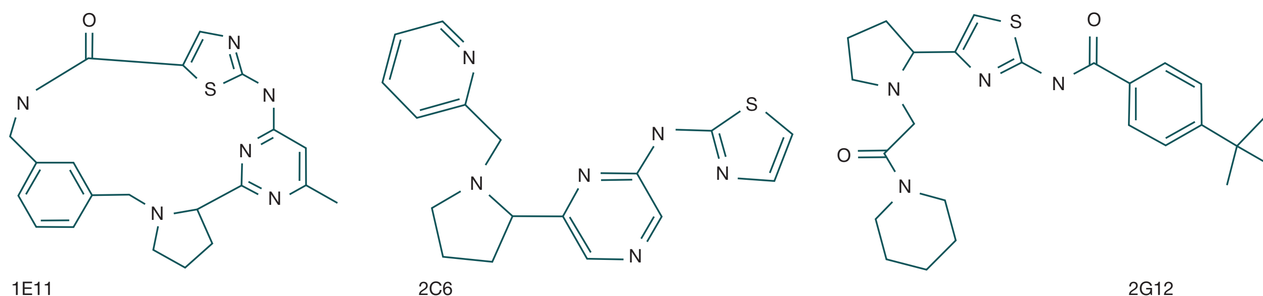

Fig. 4. Chemical structures of aminopyridine- and aminopyrimidine-based compounds selected as potential antituberculosis agents

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MIRU-VNTR GENOTYPING OF *MYCOBACTERIUM TUBERCULOSIS* CLINICAL ISOLATES FROM MOSCOW REGION

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Antibiotic selection pressure, genetic polymorphism as well as diversity of the immune status of the host and other selection factors continuously prompt *Mycobacterium tuberculosis*, the tuberculosis causative agent, to evolve. Significant or insignificant mutations shape new (sub)lineages of the pathogen whose evolution can be understood only through analyzing and monitoring its genotypic diversity and properties of its lineages. In our study we used a set of 46 *M. tuberculosis* clinical isolates from Moscow region. The samples were typed using the standard 24-loci MIRU-VNTR technique. Beijing family isolates were shown to prevail in the collection (60.9 %), as well as Beijing-B0/W148 subtype (60.7 % of total Beijing type samples); most of them (88,2 %) were multidrug-resistant resistant. The applied technique allowed us to detect one case of a mixed-strain infection.

Keywords: *Mycobacterium tuberculosis*, genotyping, phylogenetics, epidemiology, Beijing, MIRU-VNTR

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ГЕНОТИПИРОВАНИЕ КЛИНИЧЕСКИХ ИЗОЛЯТОВ *MYCOBACTERIUM TUBERCULOSIS*, ВЫДЕЛЕННЫХ В МОСКОВСКОМ РЕГИОНЕ, МЕТОДОМ MIRU-VNTR

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Под воздействием различных селективных факторов (применение антибиотиков, генетический полиморфизм и разнообразие иммунных статусов хозяина и др.) возбудитель туберкулеза *Mycobacterium tuberculosis* постоянно эволюционирует. Возникают новые линии и сублинии, характеризующиеся набором значимых и незначимых мутаций. Анализ и мониторинг представленности различных линий и их особенностей является важным для понимания эволюции патогена. В данной работе была использована коллекция из 46 клинических изолятов *M. tuberculosis*, выделенных в Московском регионе. Была определена их генотипическая принадлежность к различным линиям и сублиниям типированием по 24 локусам MIRU-VNTR. Было показано преобладание изолятов линии Beijing в коллекции (60,9 %) и изолятов сублинии Beijing-B0/W148 (60,7 % внутри линии Beijing), характеризующихся множественной лекарственной устойчивостью (88,2 % изолятов в данной выборке). Также использованный метод позволил определить один предполагаемый случай смешанной инфекции.

Ключевые слова: *Mycobacterium tuberculosis*, генотипирование, филогенетика, эпидемиология, Beijing, MIRU-VNTR

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Drug resistance of *Mycobacterium tuberculosis*, the causative agent of tuberculosis, is a major issue in the treatment of this infection. In Russia its annual incidence is estimated as 80 cases per 100,000 population (or a total of 115,000 cases per year). In 20 % of new cases and 50 % of relapses reported in Russia, patients are infected with multidrug-resistant (MDR) strains [1]. Therefore, improvement of treatment strategies largely relies on the identification and study of the most prevalent *M. tuberculosis* strains circulating in the country.

M. tuberculosis population can be divided into a number of major lineages; each lineage is geographically associated [2] and carries certain phylogenetic markers that shape the phenotype of the strain [3]. Members of the Beijing family are the most prevalent lineage in Russia; they are highly transmissible

and virulent, have a higher mutation rate and other properties contributing to their dissemination [4].

Recent research conducted in Russia [5] identified a Beijing-B0/W148 variant of the Beijing lineage. These strains exhibit increased virulence in comparison with the progenitor Beijing family and are multidrug-resistant (there are almost no drug-sensitive strains within this sublineage). Mokrousov et al. called Beijing-B0/W148 "a successful clone" of *M. tuberculosis* [5].

The lineage of the *M. tuberculosis* strain/isolate can be determined using a variety of genotyping methods, such as the IS6110-based restriction fragment length polymorphism (RFLP) analysis, spoligotyping [6], differentiation based on the use of single nucleotide polymorphisms (SNPs) of housekeeping genes [7] and type II toxin–antitoxin systems [8]. These methods are different in terms of labor intensity, cost and their discriminatory power. One of the fastest and cheapest methods that nevertheless has a good discriminatory ability is molecular genotyping based on the variable number tandem repeat analysis targeting mycobacterial interspersed repetitive units (MIRU-VNTR) [9].

Previously we analyzed a collection of 64 *M. tuberculosis* isolates from patients of the Central Research Institute for Tuberculosis, Moscow. Spoligotyping revealed that 70.3 % of the isolates belonged to the Beijing lineage [10]. To estimate the proportion of "successful clones" (Beijing-B0/W148) among Beijing strains and to identify the phylogenetic structure across the collection, we genotyped 46 DNA samples using 24-loci MIRU-VNTR. Results are presented below.

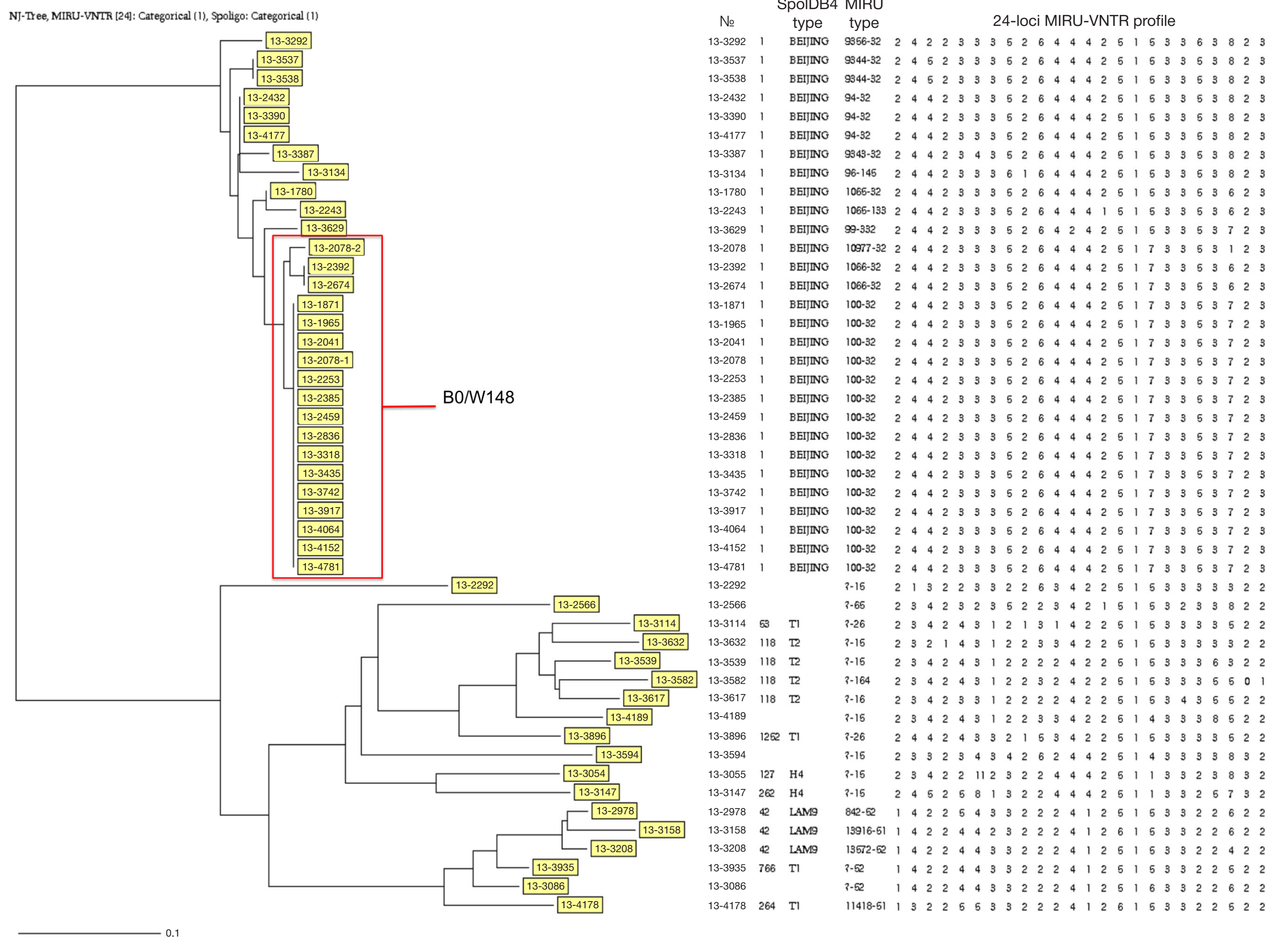
METHODS

Collection of DNA samples of M. tuberculosis clinical isolates

We used a collection of DNA samples of *M. tuberculosis* clinical isolates previously described by Maslov et al. [10]. We have previously spoligotyped the isolates and prepared their drug-resistance profiles using 8 first- and second-line antituberculosis drugs. Then the isolates were distributed into two groups: 1) isolates resistant to any of the antituberculosis drug used in the study (n = 41); 2) controls — drug-sensitive isolates (n = 23). In total, 46 isolates were analyzed (23 from each group).

Genoptyping of M. tuberculosis clinical isolates

Genotyping was performed based on 24 MIRU-VNTR loci according to the standard protocol [11]. PCR primers were synthesized by Syntol, Russia. Amplification was performed in 0.2 ml 96-well plates (Bio-Rad, USA) using the Amplification Kit (Dialat, Russia) according to the protocol described in [9] in the T100 Thermal Cycler (Bio-Rad). The obtained fragments were separated by 2 % agarose gel electrophoresis in the 1x Tris-



№		SpolDB4 type	MIRU type	24-loci MIRU-VNTR profile
13-3292	1	BEIJING	9356-32	2 4 2 2 3 3 3 5 2 6 4 4 4 2 5 1 5 3 3 6 3 8 2 3
13-3537	1	BEIJING	9344-32	2 4 5 2 3 3 3 5 2 6 4 4 4 2 5 1 5 3 3 5 3 8 2 3
13-3538	1	BEIJING	9344-32	2 4 5 2 3 3 3 5 2 6 4 4 4 2 5 1 5 3 3 5 3 8 2 3
13-2432	1	BEIJING	94-32	2 4 4 2 3 3 3 5 2 6 4 4 4 2 5 1 5 3 3 5 3 8 2 3
13-3390	1	BEIJING	94-32	2 4 4 2 3 3 3 5 2 6 4 4 4 2 5 1 5 3 3 5 3 8 2 3
13-4177	1	BEIJING	94-32	2 4 4 2 3 3 3 5 2 6 4 4 4 2 5 1 5 3 3 5 3 8 2 3
13-3387	1	BEIJING	9343-32	2 4 4 2 3 4 3 5 2 6 4 4 4 2 5 1 5 3 3 5 3 8 2 3
13-3134	1	BEIJING	96-145	2 4 4 2 3 3 3 6 1 6 4 4 4 2 5 1 5 3 3 5 3 8 2 3
13-1780	1	BEIJING	1065-32	2 4 4 2 3 3 3 5 2 6 4 4 4 2 5 1 5 3 3 5 3 6 2 3
13-2243	1	BEIJING	1065-133	2 4 4 2 3 3 3 5 2 6 4 4 4 1 5 1 5 3 3 5 3 6 2 3
13-3629	1	BEIJING	99-332	2 4 4 2 3 3 3 5 2 6 4 2 4 2 5 1 5 3 3 5 3 7 2 3
13-2078	1	BEIJING	10977-32	2 4 4 2 3 3 3 5 2 6 4 4 4 2 5 1 7 3 3 5 3 1 2 3
13-2392	1	BEIJING	1066-32	2 4 4 2 3 3 3 5 2 6 4 4 4 2 5 1 7 3 3 5 3 6 2 3
13-2674	1	BEIJING	1066-32	2 4 4 2 3 3 3 5 2 6 4 4 4 2 5 1 7 3 3 5 3 6 2 3
13-1871	1	BEIJING	100-32	2 4 4 2 3 3 3 5 2 6 4 4 4 2 5 1 7 3 3 5 3 7 2 3
13-1965	1	BEIJING	100-32	2 4 4 2 3 3 3 5 2 6 4 4 4 2 5 1 7 3 3 5 3 7 2 3
13-2041	1	BEIJING	100-32	2 4 4 2 3 3 3 5 2 6 4 4 4 2 5 1 7 3 3 5 3 7 2 3
13-2078	1	BEIJING	100-32	2 4 4 2 3 3 3 5 2 6 4 4 4 2 5 1 7 3 3 5 3 7 2 3
13-2253	1	BEIJING	100-32	2 4 4 2 3 3 3 5 2 6 4 4 4 2 5 1 7 3 3 5 3 7 2 3
13-2385	1	BEIJING	100-32	2 4 4 2 3 3 3 5 2 6 4 4 4 2 5 1 7 3 3 5 3 7 2 3
13-2459	1	BEIJING	100-32	2 4 4 2 3 3 3 5 2 6 4 4 4 2 5 1 7 3 3 5 3 7 2 3
13-2836	1	BEIJING	100-32	2 4 4 2 3 3 3 5 2 6 4 4 4 2 5 1 7 3 3 5 3 7 2 3
13-3318	1	BEIJING	100-32	2 4 4 2 3 3 3 5 2 6 4 4 4 2 5 1 7 3 3 5 3 7 2 3
13-3435	1	BEIJING	100-32	2 4 4 2 3 3 3 5 2 6 4 4 4 2 5 1 7 3 3 5 3 7 2 3
13-3742	1	BEIJING	100-32	2 4 4 2 3 3 3 5 2 6 4 4 4 2 5 1 7 3 3 5 3 7 2 3
13-3917	1	BEIJING	100-32	2 4 4 2 3 3 3 5 2 6 4 4 4 2 5 1 7 3 3 5 3 7 2 3
13-4064	1	BEIJING	100-32	2 4 4 2 3 3 3 5 2 6 4 4 4 2 5 1 7 3 3 5 3 7 2 3
13-4152	1	BEIJING	100-32	2 4 4 2 3 3 3 5 2 6 4 4 4 2 5 1 7 3 3 5 3 7 2 3
13-4781	1	BEIJING	100-32	2 4 4 2 3 3 3 5 2 6 4 4 4 2 5 1 7 3 3 5 3 7 2 3
13-2292			?-15	2 1 3 2 2 3 3 2 2 6 3 4 2 2 5 1 5 3 3 3 3 3 2 2
13-2566			?-65	2 3 4 2 3 2 3 5 2 2 3 4 2 1 5 1 5 3 2 3 3 8 2 2
13-3114	53	T1	?-26	2 3 4 2 4 3 1 2 1 3 1 4 2 2 5 1 5 3 3 3 3 5 2 2
13-3632	118	T2	?-15	2 3 2 1 4 3 1 2 2 3 3 4 2 2 5 1 5 3 3 3 3 3 2 2
13-3539	118	T2	?-15	2 3 4 2 4 3 1 2 2 2 2 4 2 2 5 1 5 3 3 3 6 3 2 2
13-3582	118	T2	?-164	2 3 4 2 4 3 1 2 2 3 2 4 2 2 5 1 5 3 3 3 5 5 0 1
13-3617	118	T2	?-16	2 3 4 2 3 3 1 2 2 2 2 4 2 2 5 1 5 3 4 3 5 5 2 2
13-4189			?-15	2 3 4 2 4 3 1 2 2 3 3 4 2 2 5 1 4 3 3 3 8 5 2 2
13-3896	1262	T1	?-26	2 4 4 2 4 3 3 2 1 5 3 4 2 2 5 1 5 3 3 3 3 5 2 2
13-3594			?-15	2 3 3 2 3 4 3 4 2 6 2 4 4 2 5 1 4 3 3 3 3 8 3 2
13-3055	127	H4	?-15	2 3 4 2 2 11 2 3 2 2 4 4 4 2 5 1 1 3 3 2 3 8 3 2
13-3147	262	H4	?-15	2 4 5 2 5 8 1 3 2 2 4 4 4 2 5 1 1 3 3 2 5 7 3 2
13-2978	42	LAM9	842-52	1 4 2 2 5 4 3 3 2 2 2 4 1 2 5 1 5 3 3 2 2 6 2 2
13-3158	42	LAM9	13916-51	1 4 2 2 4 4 2 3 2 2 2 4 1 2 6 1 5 3 3 2 2 6 2 2
13-3208	42	LAM9	13572-52	1 4 2 2 4 4 3 3 2 2 2 4 1 2 5 1 5 3 3 2 2 4 2 2
13-3935	766	T1	?-52	1 4 2 2 4 4 3 3 2 2 2 4 1 2 5 1 5 3 3 2 2 5 2 2
13-3086			?-52	1 4 2 2 4 4 3 3 2 2 2 4 1 2 5 1 6 3 3 2 2 6 2 2
13-4178	264	T1	11418-51	1 3 2 2 5 5 3 3 2 2 2 4 1 2 6 1 5 3 3 2 2 5 2 2

NJ phylogenetic tree of *M. tuberculosis* isolates from the Moscow region. The tree was constructed using the 24-loci MIRU-VNTR profile of each phylogenetic group. Beijing-B0/W148 is shown in red

acetate-EDTA (TAE) buffer (40 mM Tris-acetate, 1 mM EDTA, pH 7.6). Results were analyzed using the MIRU-VNTR*plus* web tool [9, 12].

RESULTS

According to the MIRU-VNTR profiles prepared using the MIRU-VNTR*plus* web tool, 60.9 % of isolates belonged to the Beijing lineage, 13.0 % — to LAM, 13.0 % — to T1 and T2, 4.3 % — to URAL, 2.2 % — to Cameroon, S and NEW-1 (one isolate per each lineage). One isolate's lineage could not be identified. Isolate 13-2078 was found to have two allelic variants of the QUB26 locus (1 and 7), which may indicate a mixed-strain infection [13].

Based on the MIRU-VNTR profiles, we constructed a dendrogram (see Figure). It clearly shows a cluster of 17 B0/W148 isolates (isolate 13-2078 is a combination of two strains, but both of them belong to the B0/W148 sublineage) accounting for 60.7 % of all Beijing strains. It should be noted that all of those strains were drug-resistant (group A); 15 of them (88.2 %) were multidrug-resistant, of which 3 (20.0 %) exhibited extensive drug resistance (XDR).

DISCUSSION

In our previous work [10] we genotyped isolates of *M. tuberculosis* by spoligotyping. Based on the obtained results, the isolates were distributed into 6 groups: 60.9 % belonged to Beijing family, 21.7 % — to T1 and T2, 6.5 % — to LAM9, 6.5 % — to H4 (proportions are specified for 46 isolates studied in this work). Five isolates had a unique genotype [10]. It might be due to accidental spacer deletions or insertions, which are quite typical for the studied gene region due to its high variability.

Analysis of 24 MIRU-VNTR loci allowed us to update the data obtained previously. Thus, we distributed 4 isolates that had been earlier assigned to the T-cluster into 3 lineages: 2 belonged to LAM, one to S and one to Cameroon). Of 3 isolates previously identified as belonging to the H4 lineage, 2 were now assigned to the Ural lineage and 1 — to NEW-1).

We also managed to identify representatives of the Beijing B0/W148 lineage among the isolates of the Beijing family. Therefore, we conclude that MIRU-VNTR typing provides a higher resolution and is capable of identifying mixed-strain infections meaning that it should be preferred over spoligotyping. Still, the best results can be obtained only when combining various genotyping techniques.

Typically, all Beijing-B0/W148 isolates were drug-resistant (88.2 % were MDR), which agrees with the data obtained earlier [5, 14]. This proves the "success" of the Beijing-B0/W148 sublineage. However, the question remains about the factors that promote selection of this particular phylogenetic group. Perhaps, increased mutational variability resulted in the functional rearrangements that allowed the strains to enhance their virulence and improve survival [4]. Further research is necessary to elucidate this question.

CONCLUSIONS

Assessment and epidemiologic control of the dissemination of successful *M. tuberculosis* lineages are crucial for the effective diagnosis and treatment of patients with tuberculosis. The results obtained in this study indicate a tendency for increasing dissemination of the Beijing-B0/W148 strains that have a typical MRD phenotype, provide an update of the current epidemiologic data for the central part of Russia and emphasize the importance of combining various genotyping methods for a comprehensive profile of *M. tuberculosis* clinical isolates.

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MANAGEMENT OF THE HIV-POSITIVE PREGNANT PATIENT WITH MARKED IMMUNODEFICIENCY AND MULTIPLE COMORBIDITIES

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Increasing HIV prevalence among women and growing numbers of HIV-positive patients who choose to become pregnant prompt a discussion of management strategies applied to such patients. In this work we analyze a case of a pregnant HIV-positive woman with marked immunodeficiency who started seeking medical advice only after she had developed severe life-threatening secondary conditions. We look at the progression of comorbidities that led to the death of the patient and her baby and evaluate the chosen treatment plan. We also propose recommendations for the management of patients with similar pathologies that include psychological care, vigilance against possible atypical progression of a comorbidity, such as tuberculosis, and extensive diagnostic evaluation.

Keywords: HIV-infection, pregnancy, expressed immunodeficiency, secondary diseases, tuberculosis

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ВЕДЕНИЕ БЕРЕМЕННОЙ ПАЦИЕНТКИ С ВИЧ-ИНФЕКЦИЕЙ И МНОЖЕСТВЕННЫМИ ВТОРИЧНЫМИ ЗАБОЛЕВАНИЯМИ НА ФОНЕ ВЫРАЖЕННОГО ИММУНОДЕФИЦИТА

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Возрастающее количество женщин, больных ВИЧ-инфекцией, и рост числа беременностей и родов среди них обусловливают актуальность изучения нетипичных случаев ведения таких пациенток. В настоящей статье проанализированы причины, приведшие к возникновению ситуации, когда беременная женщина с ВИЧ-инфекцией и выраженным иммунодефицитом обратилась за медицинской помощью только при возникновении тяжелых, опасных для жизни вторичных заболеваний. Рассмотрены особенности течения сочетанной с беременностью патологии, приведшей к гибели ребенка и матери. Дана оценка тактики ведения больной, разработаны рекомендации по ведению пациенток с подобными патологиями, а именно: обязательное психологическое сопровождение, настороженность в отношении возможного атипичного течения многих заболеваний, в частности, туберкулеза, максимальное расширение диагностического поиска.

Ключевые слова: ВИЧ-инфекция, беременность, выраженный иммунодефицит, вторичные заболевания, туберкулез

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In recent years, Russia has seen an increase in the number of HIV transmissions in heterosexual sexual contacts, and therefore the share of HIV-positive women grows. In this connection, the problem of pregnancy and HIV combined in one body becomes more urgent: more and more pregnant women from well-off families bear children and more and more HIV-positive women decide against abortion [1–3].

Treating such patients presents certain difficulties. Doctors need to reduce the risk of transplacental transmission of HIV and to keep the expectant mother relatively healthy. Moreover, the woman needs not only to give birth to a healthy baby but also to be able to bring it up. Therefore, women with HIV undergo highly active antiretroviral treatment with the most advanced, effective and safe drugs and methods [1, 4–6].

Pregnancy is a difficult period in life of any woman, both from physiological and psychological points of view. This is when a woman is emotionally vulnerable and helpless and needs special care and support from others. HIV adds a number of mental state peculiarities and motives to safe the pregnancy to the mix [7]. In particular, research shows that HIV-positive pregnant women treat themselves negatively, believe their social role is low and have conflicting identities that make up the self-concept [8]. In addition, pregnancy in the presence of HIV often results in complications such as preeclampsia, chronic

intrauterine fetal hypoxia, anemia, threatened miscarriage, fetal growth retardation syndrome. For example, a research led by Yatsenko [9] shows that despite ongoing antiretroviral therapy (ART) such conditions were observed in 23.5, 55.0, 23.7, 20.0 and 7.0 % of cases, respectively. HIV-positive women also often deliver preterm and suffer from labor abnormalities. Surgical abdominal delivery, while reducing the likelihood of intrapartum HIV transmission to the child, significantly increases the risk of postpartum sepsis and boosted HIV development in mother's body [10]. Secondary diseases pose special problems. If a woman has a profound immunodeficiency, such diseases threaten both the life of the child and that of her own. Secondary infections are hard to treat, since the drugs chosen need to have the least negative impact on the fetus [11–14].

All in all, treatment strategy for HIV-positive pregnant women is well developed [1, 5, 6, 15, 16]. Its most cases, application of this strategy leads to the birth of a healthy child without harming the mother's health [1, 17]. But there are some complex cases that do not necessarily work out well. Below is a description of one of such cases.

Case description

Patient U., 31 years old, was admitted to hospital infection ward on May 28, 2016, complaints: fever up to 39 °C, weakness, malaise, cough with little phlegm, shortness of breath, loss of appetite, weight loss (6 kg per month), sweating.

Anamnesis shows the acute stage began in late April: fever, body temperature up to 39 °C, dry cough. The patient tried treating the condition with symptomatic drugs, to no effect. May 20–24 2016, patient was treated in hospital following the "community-acquired pneumonia" diagnosis: ceftriaxone, 2 g per day, intramuscularly and pathogenetic therapy. The treatment resulted in a slight improvement but the fever persisted. The patient left the hospital without permission. After 2 days, she started suffering from shortness of breath, fatigue and malaise became worse and May 28 the patient once again came to the hospital and was hospitalized.

Through the q&a session is became clear that patient was pregnant, 14 weeks, but she was not registered with the women consultancy. It was the first pregnancy, desired and in a marriage. In addition, in 2005 the patient was diagnosed with HIV, but, according to her, the hospital staff treated her dismissively and rudely so she reacted negatively and has grown afraid of medical care. Thus, she did not come monitoring examinations all these years and did not receive treatment. In 2015, before the marriage, the patient underwent HIV testing anonymously. According to her, the result was negative, but the report read "Reference value: "negative "; result of HIV RNA PCR: 104,607 copies/ml." That means the patient interpreted the results erroneously. She also had 4 surgeries for congenital abnormalities (cleft palate and lip), the last of which happened in 2005.

The patient's epidemiological anamnesis shows no aggravations.

When admitted to the hospital, her condition was considered moderately grave. The body mass was short (height — 169 cm, weight — 42 kg, BMI = 14.7 kg/m^2). Pale skin, mucous oropharyngeal hyperemia, granular back wall. Lymph nodes (submandibular, cervical, axillary, inguinal) enlarged to 0.7 cm, painless, mobile. Respiratory system: sounds from lungs (percussion), hard breathing (auscultation), weak in lower sections, no wheezing. Muffled heart sounds. Coated tongue. Abdomen increased in size (due to pregnancy). Other organs and systems - no abnormalities. CBC revealed signs of inflammation: neutrophilia (92 %), lymphopenia (6 %), high erythrocyte sedimentation rate (75 mm/h). Blood chemistry analysis revealed hypoproteinemia, small-scale hypertransaminasemia. UA revealed mild case of proteinuria (hereinafter, test results are shown on the figure). The diagnosis: "Viral infection, unspecified, moderately grave. Community-acquired pneumonia? Illness caused by HIV, with manifestations of infectious diseases. Stage 4B, progression without ART. Generalized lymphadenopathy. Cachexia. Pregnancy — 14 weeks." Patient received ceftriaxone 2 g per day intravenously and pathogenetic treatment (incl. infusion).

On May 30, patient's condition deteriorated, she suffered from aggravated shortness of breath, weakness. Liver size increased 2.5 cm. Glucocorticosteroids (dexamethasone 8 mg per day) were added to the treatment plan.

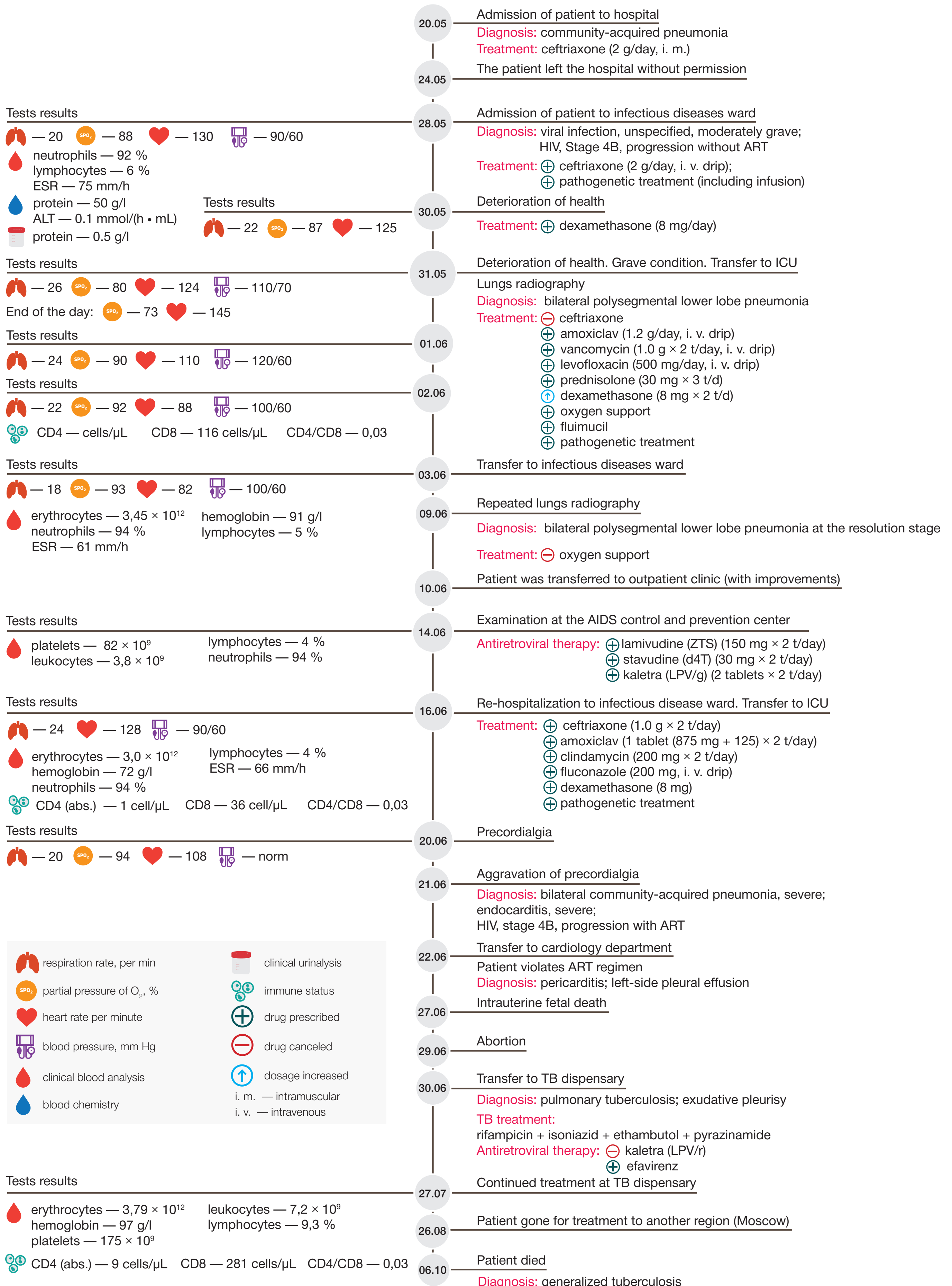
Next day, May 31, brought no alleviation: the patient was short of breath even at rest, suffered from rare dry cough and pains in the left part of the chest. Her condition was regarded as grave. Based on the chest radiograph, the diagnosis was bilateral polysegmental lower lobe pneumonia. Clarified clinical diagnosis was "Illness caused by HIV, with manifestations of bacterial and fungal infections. Stage 4B, progression without ART. Bilateral community-acquired polysegmental pneumonia (pneumocystis?), severe course. RF1. Cachexia. Orofacial candidiasis. Pregnancy — 14 weeks." Changes in the treatment plan: ceftriaxone canceled, prescribed: amoksiklav, vancomycin, levofloxacin, increased doses of corticosteroids, oxygen support, salbutamol inhalations, expectorants (Fluimucil), intensified infusion therapy (dosage and methods of administration are hereinafter shown on the figure). Additional tests were ordered, including sputum collection (no success). Through the day, the patient's condition continued to deteriorate: SpO_2 — 73 %, heartbeat — 145 BPM, urine output decreased to 500 ml/day. Aggravating cardiopulmonary failure brought the patient to intensive care unit.

June 1: patient complains about weakness and cough; condition remains grave but the trend is positive. June 2: body temperature normalized at 36.4 °C. Immunity and virus tests returned as follows: CD4$^+$ T-lymphocytes — 4 cells/μL, CD8$^+$ T-lymphocytes — 116 cells/μL, CD4/CD8 — 0.03; HIV RNA PCR — 1615358 copies/μL. Tests for markers of viral hepatitis returned negative. Additional consultations given by obstetrician-gynaecologist ("Pregnancy, 14 weeks. No indications for abortion") and phthisiologist ("More data supports bilateral nonspecific pneumonia"). Prescribed: tuberculin skin tests and sputum collection to find Mycobaterium tuberculosis (3 times). On June 3 the patient was transferred to the infectious disease ward. Sputum study was not performed due to lack of material.

On June 9 patient began complaining about cough and weakness again. Vital signs were normal, including body temperature. Repeated chest radiograph was the basis for diagnosed bilateral polysegmental lower lobe pneumonia at the resolution stage. Oxygen support was canceled. The next day, following the request of the patient she was transferred to outpatient clinic (with improvements).

On June 14 U. was examined by an infectiologist at the AIDS control and prevention center. The patient complained of dry cough, weakness and body temperature up to 38.5 °C from June 11. Gynecologist confirmed the patient was 16–17 weeks pregnant. CBC revealed thrombocytopenia, leukopenia, lymphopenia and neutrophilia. The patient was prescribed ART (figure). On June 15, her body temperature rose to 40 °C. U. was re-hospitalized at infectious disease ward.

Her condition was regarded as grave when she arrived there. Conditions found at the moment: tachycardia and



Brief clinical record of U., 31, female, pregnant, HIV-positive

low blood pressure; 2 cm liver protrusion from under the costal arch; decreased urine output; anemia, neutrophilia, lymphopenia; increasing lab immunodeficiency. The patient was transferred to the intensive care unit. Prescribed antibiotics — ceftriaxone, amoxiclav, clindamycin, antifungal fluconazole, dexamethasone, pathogenetic drugs. In the following days, the body temperature returned to normal, vital signs were stable, partial oxygen pressure was normal.

June 20: patient started feeling pains in the left part of her chest, body temperature increased again (38.2–38.6 °C). Electrocardiogram revealed sinus tachycardia. The next day, the pains in the chest became stronger. Chest radiograph was the basis for diagnosed bilateral polysegmental lower lobe pneumonia at the resolution stage. Diagnosis: "Bilateral community-acquired pneumonia, severe course. RF1. Infective endocarditis, severe course. Cardiopulmonary failure, stage I–II. Illness caused by HIV, with manifestations of bacterial, fungal infection, stage 4B, progression with ART in the background. Generalized lymphadenopathy. Cachexia. Expressed lab immunodeficiency. Hypochromic anemia. Pregnancy — 16–17 weeks." On June 22, the patient was transferred to cardiology department of somatic ward where the diagnosis was clarified: "HIV-infection, stage 4B, progression with ART in the background. Generalized lymphadenopathy. Bilateral polysegmental pneumonia, severe course. Pericarditis. Left-side pleural effusion. Body mass deficiency over 30 %. Cachexia. Expressed lab immunodeficiency. Hypochromic anemia. Thrombocytopenia. Pregnancy — 16–17 weeks." The patient continued ART course but complained about poor tolerance and was lax with the schedule.

On June 27, ultrasound revealed the fetus was dead. On June 29, the pregnancy was terminated (17–18 weeks). The patient continues to suffer from fever, leukocytosis increases in the blood because of stab cells.

Diagnosis of June 30: "Pulmonary tuberculosis. Pleural effusion." The patient was transferred to TB dispensary where she was prescribed first regimen specific therapy (rifampicin + isoniazid + ethambutol + pyrazinamide). Rifampicin forced a change in ART course: lopinavir/ritonavir was canceled and efavirenz prescribed.

On July 27, the patient continues to receive treatment in the TB dispensary. Clinical diagnosis: "HIV, stage 4B, remission with ART in the background. Disseminated pulmonary tuberculosis, infiltration and decay phase. AFB (–). Tuberculosis polyserositis. Pericarditis. Right-side exudate tuberculosis pleurisy. AFB (+) in exudate. Tuberculous papillitis. AFB (+) in urine. IA (MTB +), dispensary registry group, pneumocystis pneumonia in anamnesis. Persistent oral candidiasis. Expressed lab immunodeficiency." Immunity and resistance to virus-induced effects show a positive trend.

On August 26 the patient was reported to move to another region to seek treatment there (city of Moscow). Therefore, any further data on her disease are not available. However, patient's husband later sent a note stating U. died on October 6. Postmortem diagnosis: "Generalized tuberculosis." Diagnosis according to ICD X: "Illness caused by HIV, with manifestation of mycobacterial infection."

Case discussion

The case described above is not a typical one. From the social status point of view, the patient was quite well off, yet, being aware of her HIV status, she not only refused routine monitoring but postponed applying for medical assistance when it was needed direly. Denial, which is a natural psychological reaction to expect from a person being informed of a positive HIV test (and which typically resolves into acceptance), stayed with U. for many years. Obviously, the reason behind the situation here is the first experience U. had with doctors, which was negative. Unfortunately, HIV-positive patients often suffer from the same attitude expressed by medical professionals. Our data indicates that up to 64 % of people living with HIV have faced unethical behavior of physicians and 21 % have to bear it often [18].

Same factor may have also contributed to the patient's refusal to register her pregnancy with the women consultancy, which is one of the reasons of its termination. Legislation secures the right of a woman to make decision about motherhood on her own. An HIV-positive woman, like any other, can give birth is she wants to [19]. But HIV imposes certain obligations on her: to bear a healthy baby, she must adhere to recommendations and, above all, take antiretroviral drugs [1, 5, 6]. In the case considered, while the pregnancy, though late, was conscious and desired, the woman was married, she violated the drug regimen. To improve her obedience to the ART regimen, a psychologist should have been invited to counsel U. Interdisciplinary approach to treatment of such patients implies having a psychologist on the team [1]. This patient's psycho-emotional problems were obvious and she needed psychological counseling. Unfortunately, hospitals rarely employ specialists of this profile. The patient received psychological support from her husband, physicians, employees of the medical university.

We believe that the ART regimen chosen in this case was justified. It was necessary not only to prevent vertical transmission of HIV, but also save life of the patient taking into account the marked immunodeficiency ($CD4^+$ T lymphocytes — 4 cells/μL). One of the regimens that work well with pregnant women (including zidovudine, lamivudine and lopinavir/ritonavir) could not be applied due to anemia. That is why zidovudine was substituted with stavudine. This regimen belongs to the ART concept used for patients with initially low (less than 50 cells/μL) number of $CD4^+$ T-lymphocytes. Choosing lopinavir over efavirenz, which the patient could not tolerate, was quite acceptable, since at this point U. was already in the 2nd trimester of her pregnancy. However, considering the fact that the patient also suffered from tuberculosis and had a low number of $CD4^+$ (less than 100 cells/μL) from the outset, national guidelines for dispensary observation and treatment of HIV-positive patients could be followed and a fourth drug, enfuvirtide, added to the mix. It would not harm the fetus but could boost the effectiveness of ART [5, 6].

Pregnancy made the already complicated situation even worse. We believe that hormonal changes associated with pregnancy could exacerbate immunodeficiency, which resulted in further development of secondary infections. The most dangerous of them turned out to be tuberculosis, which lead to the death of the patient.

As is well known, tuberculosis takes an unusual course when there is immunodeficiency in the background [1, 20–22]. With $CD4^+$ T-lymphocytes content below 500 cells/μL, the disease develops faster, its destructive forms are more difficult to identify, serous membranes get involved in the pathological process more often. When the content of $CD4^+$ T-lymphocytes is below 350 cells/μL, pulmonary tuberculosis is associated with damaged pleura, pericardium, when it is below 200 cells/μL, there can be miliary lymphogenous dissemination with multiple organ lesions expressed through devastating syndrome, fever [1]. All of these symptoms have been observed in patient U. With profound immunodeficiency, (less than 100 $CD4^+$ T-lymphocytes/μL) tuberculosis is often (up to 40 % of cases)

accompanied by development of other secondary diseases, which makes it even more difficult to make a diagnosis. Due to the almost complete loss of signs of granulomatous inflammation, in such patients tuberculosis morphologically and clinically resembles pneumonia. In 30 % of cases the clinical picture is 4–8 weeks ahead of dissemination, and in some patients radiography reveals no changes at all [1]. The case in question is a perfect example of such a turn of events. Since acute immunodeficiency makes radiography, tuberculin test and diaskintest uninformative, timely detection of TB requires morphological, direct microscopic and bacteriological, molecular genetic tests of biological fluids and biopsies. But, as this case shows, these tests also do not guarantee a quick result and sometimes simply cannot be performed. Difficulties with diagnostics and a high probability of generalization of tuberculosis when immunodeficiency is substantial indicate that patients with the $CD4^+$ T lymphocytes content below 200 cells/μL need preventive treatment [6]. We believe that at the very beginning of treatment of patient U. there were good reason to assume she suffered from respiratory system disorders of mixed etiology, including tuberculosis (despite the absence of typical signs on radiographs), and thus consider treating her ex juvantibus.

Pulmonary tuberculosis, which remained unseen in the early stages, could have been boosted by concomitant lesions of Pneumocystis etiology. Although Pneumocystis jiroveci were not found and the diagnosis was not confirmed in laboratory, clinical factors supporting presence of this pathology were gradually increasing respiratory distress (and scarcity of physical findings), high levels of ESR, low rates of the SpO_2 (to 73 %), and positive effect produced by clindamycin and glucocorticosteroids.

Serious polietiologic damage to the lungs, namely hypoxia that followed cardiopulmonary disorder, was, in our opinion, one of the main causes of fetal death. An important role was also played by general exhaustion that manifested not only as a significant reduction in body weight, but also as asthenic syndrome, anemia, hypoproteinemia. The risk of spontaneous abortion in this case can be considered very high. But even if the pregnancy was saved, the risk of serious consequences for the health of the child was considerable. According to one study, posthypoxic CNS damage of varying degrees of severity were reported in 90.5 % of children, hematological changes in the form of anemia (mild to moderate) — 97.3 % of children [23]. In the case we are considering here, the woman could not save this pregnancy, regardless of how much she desired it. The list of medical indications for abortion does not contain HIV, but in some cases, with all the risks taken into account and after a peer review, abortion is possible even after 12 weeks. On the other hand, in this case the operation can substantially harm health of the HIV-positive woman make the main diseases worse [24, 25]. In any case, it is the woman who always makes the choice. This is a difficult decision, and we believe in such situations psychological support is in order.

CONCLUSIONS

The case described above allows a number of conclusions.

Firstly, it is necessary to systematically work on making society treat HIV-positive individuals well. This is especially urgent in medical community, since such work can significantly increase the effectiveness of interactions between doctors and patients and prevent rejection of treatment by patients.

Secondly, treatment of patients with a complex mix of medical, psychological, ethical issues requires psychological support, so hospitals with infectious diseases wards should employ medics of the corresponding profile. Currently, physicians have to play the role psychologists and they do not always have the time and the necessary qualifications. As a recommendation, psychologists can be enlisted as volunteers from the circles of medical professionals, medical students (with the written consent of the patient), family members who are aware of the HIV-positive status of the individual.

Thirdly, successful treatment of HIV-positive pregnant women with severe immunodeficiency requires complete and rapid detection of secondary diseases and addressing them adequately. It is important to remember that very often immunodeficiency means that damage to organs has a number of reasons behind it and develops unusually. Doctors should always be on alert and maximize the spectrum of diagnostic search they perform and use all available methods. Treatment should be adequate. In cases when it is not possible to find the pathogen, the treatment should be empirical, with all the factors take into account. We believe that in some cases ex juvantibus therapy is a justified choice.

Finally, despite all the efforts medical professionals make, success is not always guaranteed. Pregnant women who are HIV-positive should be informed of the possible risks. To evaluate those risks, all the data available should be collected and analyzed. This task can be solved efficiently if the process of collection was automated and introduced to daily routine.

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CLINICAL, IMMUNOLOGICAL AND VIROLOGICAL INDICATORS OF ANTIRETROVIRAL THERAPY EFFICIENCY

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Antiretroviral therapy (ART) for HIV-positive patients allowed labeling the disease a therapeutically controlled one. The main goal of ART is to prolong patient's life and preserve its quality. This is accomplished through viral load reduction (decrease of the number of HIV-RNA copies in blood plasma), which leads to the growing numbers of CD4⁺-T-lymphocytes. However, ART can be ineffective. In 2010–1014, we conducted an observational cohort retro/prospective study aimed at learning how often ART can be ineffective from immunological (II), virological (VI) and clinical points of view. The study was carried out at the premises of the Republic Center of AIDS and Infectious Diseases (Kazan, Russia). The study included 341 adult HIV-positive patients subjected to ART at 3rd and 4th stages of disease's development, with the treatment virologically efficient at least during the first year. The observation period was 1 to 3 years. ART was considered II (immunologically inefficient) when the number of CD4⁺ increased for less than 50 cells/mcl through the year with HIV completely suppressed. VI (virological inefficiency) of ART was registered if the number of HIV RNA copies was above the definition threshold after 6 months of treatment. ART was II in 14.0–15.9 % of cases after a year of treatment and in 22 % of cases after three years. It was noted that subsequent restoration of an adequate number of T-lymphocytes CD4⁺ required they overcame the threshold of 100 cells/mcl within the 1st year of treatment. Virologically, ART was effective for 92.7 % for patients. Most (80 %) cases of VI of ART were results of patients' lax attitude towards treatment. Clinically, ART helped 91 % of patients; this result largely depended on the number participants for whom ART was II. II of ART is a risk factor, the risk being progression of the disease with active ART in the background and death of the HIV-positive individual. II of ART makes the risk of clinical progression of HIV 6.232 times higher (95 % CI 3.106–12.51).

Keywords: HIV, antiretroviral therapy, clinical efficacy, virological efficacy, immunological efficacy, therapy failure

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КЛИНИКО-ИММУНОЛОГИЧЕСКИЕ И ВИРУСОЛОГИЧЕСКИЕ ПОКАЗАТЕЛИ ЭФФЕКТИВНОСТИ АНТИРЕТРОВИРУСНОЙ ТЕРАПИИ

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Применение антиретровирусной терапии (АРВТ) при ВИЧ-инфекции позволило перевести заболевание в разряд терапевтически контролируемых. Основная цель АРВТ — увеличение продолжительности жизни пациента и сохранение ее качества. Она достигается снижением вирусной нагрузки (числа копий РНК ВИЧ в плазме крови), что приводит к росту числа CD4⁺-Т-лимфоцитов. Но терапия может быть неэффективной. В работе изучена частота случаев иммунологической (ИН), вирусологической (ВН) и клинической неэффективности АРВТ. В исследование включили 341 взрослого ВИЧ-инфицированного пациента, получавшего АРВТ, начатую на стадиях 3–4 заболевания, с вирусологической эффективностью как минимум в течение первого года лечения. Участников исследования наблюдали на протяжении 1–3 лет. ИН АРВТ определяли как увеличение числа клеток CD4⁺ менее чем на 50 клеток/мкл в год на фоне полной супрессии ВИЧ, ВН АРВТ — как число копий РНК ВИЧ выше порога определения через 6 мес от начала лечения. Частота случаев ИН АРВТ составила 14,0–15,9 % для одного года лечения и 22 % для трех лет наблюдений. Отмечено, что для последующего адекватного восстановления содержания Т-лимфоцитов CD4⁺ необходимо увеличение их числа в первый год терапии более чем на 100 клеток/мкл. Вирусологически эффективной АРВТ была для 92,7 % пациентов. Большая часть (80 %) случаев ВН АРВТ была обусловлена низкой приверженностью пациентов к лечению. Клиническая эффективность АРВТ составила 91 % и в значительной степени определялась числом участников исследования с ИН АРВТ. Иммунологическая неэффективность АРВТ является фактором риска прогрессирования заболевания на фоне АРВТ и смерти при ВИЧ-инфекции. Риск клинического прогрессирования ВИЧ-инфекции при ИН АРВТ выше в 6,232 раз (95 % ДИ 3,106–12,51).

Ключевые слова: ВИЧ-инфекция, антиретровирусная терапия, клиническая эффективность, вирусологическая эффективность, иммунологическая эффективность, неэффективность терапии

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The main goal of ART is to prolong patient's life and preserve its quality. Clinical ineffectiveness (CI) of ART results in development of a new opportunistic infection in the patient's body after 3–6 months of treatment. Thus, it takes a long-term study to evaluate effectiveness of ART.

Important aspects of ART that determine the rate of HIV-positive patients survival are virological and immunological responses to the therapy. From the virus control point of view, the task is to minimize the viral load in the patient's body (preferably to below 50 RNA copies/ml) and hold it there as long as possible to stop progression of the disease and prevent development of viral resistance to drugs. Virological ineffectiveness (VI) of ART increases the risk of HIV progression in several times [1, 2]. Immunologically, ART is successful when the number of CD4+ T-lymphocytes is growing (the growth is significantly more intensive with the full viral suppression). Immunological ineffectiveness (II) of ART is observed in approximately 15 % of patients [3]: they suffer from immunodeficiency for a long time despite taking antiretroviral drugs, which increases the risk of development of AIDS- and non-AIDS-defining diseases [4].

The aim of our study was to assess the incidence of clinical, virological and immunological inefficiency of ART and determination of conditions ensuring effectiveness of ART.

METHODS

The observational cohort retro/prospective study was conducted in 2010–2014 at the premises of the Republic Center of AIDS and Infectious Diseases Prevention and Treatment, Kazan (hereinafter — AIDS Center). The study was approved by the local ethics committee (Protocol 3 dated March 24, 2015).

Data used in the study described adult patients with a confirmed HIV-positive status that were in the dispensary observation at the AIDS Center in 1999–2014. Criteria for inclusion were as follows: 1) ART receiver for more than a year, HIV RNA in blood plasma below the registration threshold after 6 months from ART start and (minimum) during the first year; 2) ART started at stages 3–4 as defined by the clinical classification of HIV approved by the Health and Human Services Ministry of the Russian Federation by Order No. 166 dated 17.03.2006. Criteria for exclusion were: 1) underage; 2) ART reception for less than one year; 3) registered HIV RNA in plasma after 6 months of ART; 4) HIV in incubation, primary symptoms and terminal stages; 5) receiving both ART and antiviral therapy against viral hepatitis. Criteria for early retirement from the study were:1) cessation of ART; 2) registered viral load (HIV RNA content) that developed after the earlier achieved suppression of HIV in plasma (to determine the immunological effectiveness of ART); 3) start of viral hepatitis antiviral therapy while receiving ART; 4) death of the patient. All patients signed informed consent to examination and ART application.

The number of observed patients was 341,204 of them were male. The age of the participants was 35 (32; 40) (hereinafter, data describing the sample are given as median and interquartile range). 59.2 % of cases were parenteral infection. The participants were registered at the AIDS center for 5 years (2; 9) before treatment. Shares of HIV stages among participants: stage 3 — 34.6 %, 4A — 29.9 %, 4B — 28.4 %, 4C — 7.1 %. Content of T-lymphocytes CD4+ in peripheral blood prior to ART: 186 (120; 277) cells/μL; content of HIV RNA in plasma: 93,000 (22,750; 280,625) copies/ml.

All patients were prescribed ART in accordance with methodological recommendations issued by the Health and Human Services Ministry of the Russian Federation, 7125-RKh dated 29.12.2006, observation period — 1 to 3 years. The first line ART regimen included two nucleoside reverse transcriptase inhibitors (NRTI), the third component was non-nucleoside reverse transcriptase inhibitor (NNRTI) or protease inhibitor (PI) or integrase inhibitor (INI) (Fig. 1).

Immunological effectiveness of ART was evaluated by the increase in the number of CD4+ T-lymphocytes. The patients were divided into two groups: 1) those with an increase of less than 50 cells/μL in a year (no response to therapy); 2) those with an increase of more than 50 cells/μL in a year. Content of CD4+ T-lymphocytes was measured at least twice, time between measurements — 3 months. This was done to eliminate random error due to variability of absolute values of the indicator.

Virological effectiveness of ART was evaluated no earlier than in the second year of treatment because undetectable viral load during the first year of treatment was an inclusion criterion. VI of ART was acknowledged if the number of HIV RNA increased over 400 copies/ml up to 2010 and over 150 copies/ml in the following years. Short-term rise in the level

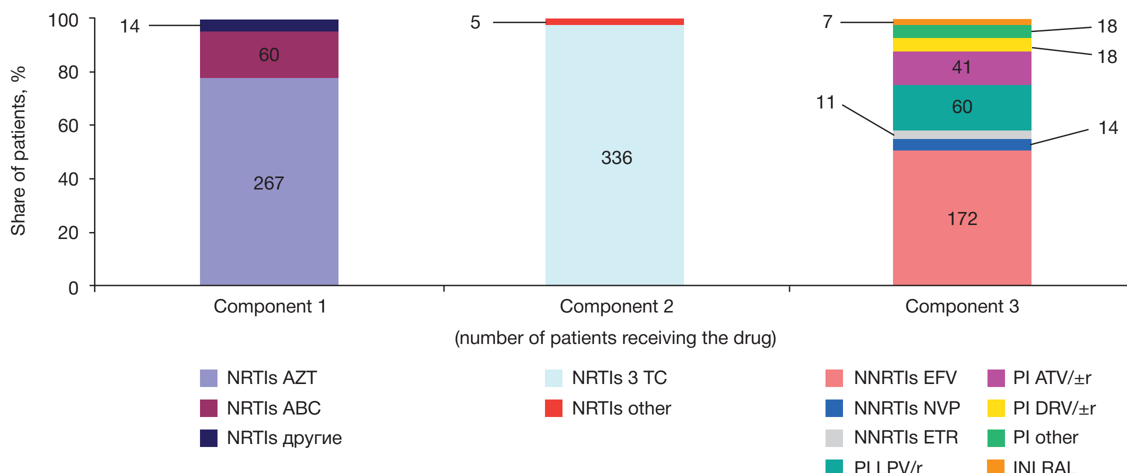

Fig. 1. Groups of patients by type of drug, three-component first line ART

NRTIs — nucleoside reverse transcriptase inhibitor; NNRTI — non-nucleoside reverse transcriptase inhibitor; PI — protease inhibitor; INI — integrase inhibitor; AZT — azidothymidine; ABC — abacavir; other NRTIs — stavudine, didanosine, phosphazide; 3TC — lamivudine; EFV — efavirenz; NVP — nevirapine; ETR — etravirine; LPV/r — lopinavir boosted with ritonavir; ATV/±r — atazanavir boosted/not boosted with ritonavir; DRV/±r — darunavir boosted/not boosted with ritonavir; other PI — fosamprenavir, saquinavir, indinavir, nelfinavir, ritonavir; RAL — raltegravir.

of HIV RNA — less than 1000 copies/ml, a "blip", — was not considered a sign therapy ineffectiveness.

CI of ART was acknowledged if a new opportunistic infection developed in the patient's body after 3–6 months of treatment. Inflammatory syndrome accompanying immune system reconstitution was not considered a sign of therapy ineffectiveness.

Content of $CD4^+$ T-lymphocytes and HIV RNA was measured before the start of ART, after 6 and 12 months after start of ART, further on — annually throughout the treatment period. Quantification of HIV RNA in plasma was performed through polymerase chain reaction in real time using Cobas Amplicor HIV-1 Monitor v.1.5 (Hoffman-La Roche, Switzerland) and LCx HIV RNA Quantitative assay (Abbott Laboratories, USA) tests in COBAS TaqMan 48 (Hoffman-La Roche) and Abbott m2000rt (Abbott Molecular, USA) analyzers with the sensitivity threshold of 400 copies/ml up to 2010 and 150 copies/ml for the following years. HIV resistance to antiretroviral drugs was determined by sequencing using ViroSeq HIV-1 Genotyping System test (Applied Biosystems, USA) with Applied Biosystems 3100 and 3130 analyzers (Applied Biosystems). The number of $CD4^+$ T-lymphocytes was determined by flow cytometry using monoclonal antibodies (mAbs). Lymphocytes phenotyping was performed by direct immunofluorescence with Multitest 6-color TBNK Reagent (Becton Dickinson, USA) containing MAb $CD3^+$, $CD4^+$, $CD8^+$, $CD16/56^+$, $CD19^+$, with account of immunofluorescence reaction in FACScanto II flow cytometer (Becton Dickinson).

A factor's influence (II of ART) on the treatment outcome (HIV disease progression, death) was measured for the entire period of therapy regardless of the time the factor appeared.

Statistical analysis of the results was performed using the methods of descriptive and comparative statistics and programs STATISTICA 10.0 (StatSoft, USA), Microsoft Excel 2003, OpenEpi 3.01 [5]. Normality of distribution was assessed with the help of the Shapiro–Wilk test. Equality of dispersions of characteristics distribution was assessed with the F-test. In case the distribution was not normal the data was presented through median and interquartile range: Me (Q1; Q3). Mann–Whitney test helped determine significance of differences between groups when comparing independent samples. Comparison of several independent groups benefited from Kruskal–Wallis H test followed by pairwise comparison of the groups using Mann-Whitney test with Bonferroni correction. Comparison of outcomes frequency was done with calculated relative risk and 95 % confidence intervals. Rejection of the null hypothesis occurred at a threshold level of statistical significance of $p = 0.05$.

RESULTS

Immunological ineffectiveness of ART

The incidence of immunological ineffectiveness of ART (II of ART) was 74 (22 %) within a 3-year period. We analyzed the timing of emergence of II of ART and dynamics of CD 4^+ T-lymphocytes growth in the future. In the first year, 48 patients of 341 patients (14 %) did not respond to ART, in the second — 41 of 261 (15.7 %), in the third — 31 out of 195 (15.9 %). See table 1 for data on dismissal of patients from the study.

Of the 48 patients that showed no response to ART in the first year, 24 remained unresponsive in the second year 24 (Fig. 2), 12 did show a response and 12 people were dismissed from the study. In addition, 17 more patients joined II of ART group in the second year. Earlier, these patients enjoyed adequate recovery of $CD4^+$ T-lymphocytes. Of these, 9 people did not respond to treatment in the third year. Thus, a significant portion of patients with II of ART (66.7 %) in the second and third years of treatment were patients that had immunological ineffectiveness of therapy developed in the first year.

During the 1st year of ART, the increase in number of $CD4^+$ T-lymphocytes was: 1) 1 (–25; 22) cells/μL in the group that showed II of ART in both the first and the second years of therapy; 2) 8 (–30; 17) cells/μL in the group that showed II of ART IN the first year and an effective restoration of $CD4^+$ T-lymphocytes numbers in the second year; 3) 102 (77; 156) cells/μL in the group that showed II of ART in the second year only; 4) 165 (83; 263)cells/μL in the group that responded to treatment both in the first and the second years of therapy ($H = 80.6$ at $p < 0.001$; $p_{12} = 0.988$; $p_{13} < 0.001$; $p_{14} < 0.001$; $p_{23} < 0.001$; $p_{24} < 0.001$; $p_{34} = 0.125$).

Of the 195 patients who were followed throughout the study, 12 (6 %) showed no response to the therapy throughout all three years ("absolute nonresponders"); 27 (13.8 %) did not respond to therapy at this or that stage of treatment ("relative nonresponders").

Virological ineffectiveness of ART

Table 1 shows that in the two years of treatment, ART was virologically ineffective (VI of ART) in 25 patients (7.3 %). Second

Table 1. Reasons for patients dismissal from the study

Reason for dismissal	Number of dismissed patients at different stages of ART							
	1st year of ART		2nd year of ART		3rd year of ART		3 years of ART. total	
	abs.	rel.. %	abs.	rel.. %	abs.	rel.. %	abs.	rel.. %
Unauthorized cessation of ART	–	–	19	5.6	11	3.2	30	8.8
Virological ineffectiveness of ART	–	–	18	5.3	7	2.1	25	7.3
Start of AVT	–	–	16	4.7	8	2.3	24	7.0
Death	–	–	7	2.1	5	1.5	12	3.5
Relocation to another region	–	–	6	1.8	2	0.6	8	2.3
Insufficient follow-up period	–	–	14	4.1	33	9.7	47	13.8
Total	–	–	80	23.5	66	19.4	146	42.8

Note. ART — antiretroviral therapy, AVT — antiviral therapy (hepatitis).

year saw more patients not responding to ART's antivirus functions than the first one: 18 patients (5.3 %) against 7 (2.0 %), espectively. For 20 (80.0 %) of 25 patients, ART was VI due to violations of regimens. Once they were helped in making a habit of taking medicines as is proper, the viral load became undetectable again and there was no need to change the regimen. For 5 (20 %) patients, picking the optimal ART regimen took studying drug-resistance of HIV. One patient had less than 1000 copies/ml of HVA RNA so picking the regimen for him was not possible. In 2 patients, the virus showed no resistance to drugs so they received further counseling aimed at forming a habit of taking medicines as prescribed. The remaining 2 patients had HIV resistant (multiple resistance) to NRTIs and NNRTIs, which made it necessary to change their regimens. Thus, VI of ART that resulted from resistance of the virus to drugs was registered in 2 (0.6 %) participants in the study.

34 patients had virus resistance to drugs analyzed before the start of ART (Table 2). 3 patients showed resistance to two classes of drugs (NRTIs/NNRTIs — 1, NRTI/PI — 2), 2 patients — to one (NNRTI). All patients with multi-resistant viruses were previously treated with drugs of the aforementioned classes, to no effect. Patients with resistance to just one class of drugs never received ART before but got the virus from partners for whom ART was VI. At that, only one patient was proved to have cross-resistant virus.

Thus, lax attitude patients show towards treatment regimens contribute significantly to ART's virological ineffectiveness. Patients for whom treatment was VI before tend to have multiple resistant viruses before the start of ART.

Clinical ineffectiveness of ART

30 patients (9 %) had HIV progressing in spite of ART. For more than half of them (62.5 %) the therapy was II. 7 patients (23 %) died due to this progression and a developed disease. Table 3 shows variants of clinical progression.

The majority of patients for whom ART was CI (clinically ineffective) had tuberculosis in 1–4 years of therapy. In 11 (61 %) cases tuberculosis was associated with ART being II. The average content of $CD4^+$ T-lymphocytes (M ± SD) associated with clinical progression of HIV in the form of TB is 277 ± 194 cells/μL, with the minimum value being 25 cells/μL and maximum — 616 cells/μL. The average content of $CD4^+$ that invited herpes infection was 262 ± 81 cells/μL, minimum and maximum values are 174 and 375 cells/μL, respectively. For Candida esophagitis, the average content of $CD4^+$ was 96 ± 41 cells/μL, for oral hairy leukoplakia — 296 cells/μL, for cryptococcosis — 63 cells/μL, for lymphoma — 13 cells/μL.

II of ART made the risk of clinical progression of HIV 6.232 times higher (95 % CI 3.106–12.51).

13 patients died during the study. Among the causes of death, TB is the leader (5 people, or 38 %), followed by diseases CVD (3 people, 23 %). The rest of the deaths were due to cryptococcosis, cirrhosis, cancer, accidents and suicide (one case). Half of the deceased patients (7 cases, 54 %) were "clinical progressors" with ART applied as prescribed. The risk of death for patients that showed no immunological response to ART was 18.6 %, while those for whom the therapy was effective ad this risk at 1.3 %. When assessing immunological ineffectiveness of ART as a death risk factor, it was found that II of ART increased patient's chances of dying 13.8 times compared to patients that had an effective increase in the number of $CD4^+$ T-lymphocytes as a result of therapy ($p < 0.001$; 95 % CI 4.359–44.07). At that, the risk of dying AIDS was 36.8–times higher ($p < 0.001$; 95 % CI 4.42–307.6).

DISCUSSION

We have found that over 3 years, ART can be II in 22 % of cases. This is greater than Russian [6] and foreign [3] colleagues reported earlier. However, considering just 1 year we received comparable results: ART was II in 14, 15.7, and 15.9 % of cases in our study, while Shmagel et al. report 11.3 % [6] and Moore et al. — 15 % [3]. To make accurate assessments, we need to monitor patients receiving ART for a long period of time because every year there may be more patients that do not respond to the therapy.

For some reason, some patients with "good" immunological response in the first year of ART did not have the number of $CD4^+$ T-lymphocytes restoring in the following years of therapy. It may be connected to peculiarities of ART regimen [7]. Another possible reason is the development of secondary diseases in the second or third years of ART, diseases that hinder restoration of $CD4^+$ number [8]. However, in the context of our study only 3 of 17 patients for whom ART was II showed clinical progression of HIV in the second year of treatment. Perhaps, the II criteria we have suggested for the 1st year of treatment should be different, i. e. the treatment can be considered effective in case there is a more pronounced immune response than 50 more $CD4^+$ cells a year [9, 10]. $CD4^+$ cells numbers increase in the course of one year of therapy indicates that addition of more than 100 cells/μL within the 1st year enhances immunologic success of the therapy, and if the number is less than 50 cells/μL, in most cases (but not always) further restoration of T-lymphocytes will be ineffective.

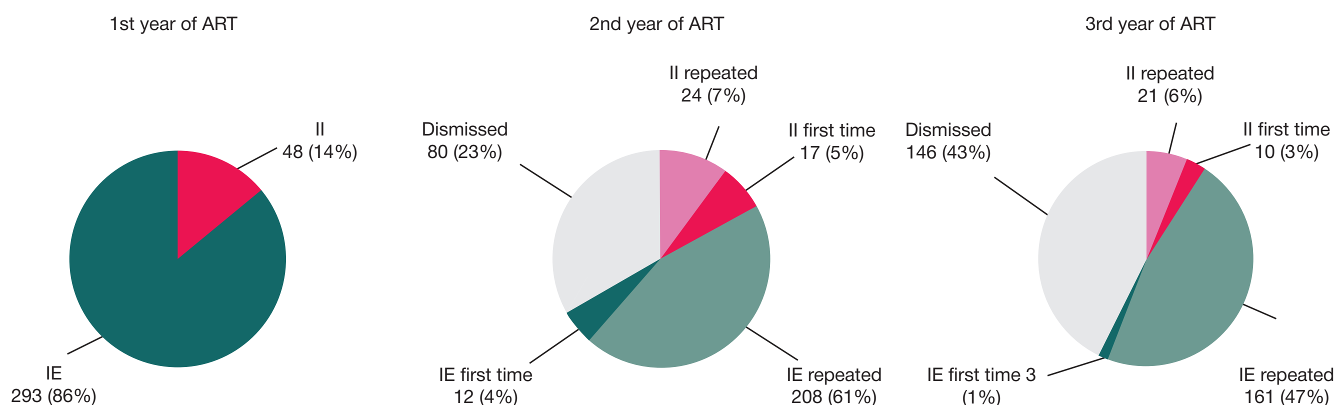

Fig. 2. Groups of patients by immunological response to ART (II — immunological ineffectiveness, IE — immunological effectiveness)

Table 2. HIV resistance to antiretroviral drugs, test results (n = 34)

HIV resistance to ART	Number of patients with HIV resistant to ART drugs.																	
Detected	5 (15 %)																	
	NRTIs							NNRTIs				PI						
	AZT	3TC	ABC	TDF	FTC	ddI	d4T	EFV	NVP	ETR	DLV	LPV/r	DRV	ATV	SQV	FPV	IDV	NFV	TPV
	2	3	2	1	3	2	1	2	2	–	1	1	–	2	1	1	1	1	–
Not detected	29 (85 %)																		

Note. ART — antiretroviral therapy; NRTIs — nucleoside reverse transcriptase inhibitor; NNRTI — non-nucleoside reverse transcriptase inhibitor; PI — protease inhibitor; AZT — azidothymidine; ABC — abacavir; 3TC — lamivudine; TDF — tenofovir; FTC — emtricitabinum; ddI — didanosinum; d4t — stavudinum; EFV — efavirenz; NVP — nevirapine; ETR — etravirine; DLV — delavirdine; LPV/r — lopinavir boosted with ritonavir; DRV/±r — darunavir boosted/not boosted with ritonavir; ATV — atazanavirum; SQV — saquinavirum; FPV — fosamprenavirum; IDV — indinavirum; NFV — nelfinavirum; TPV — tipranavirum.

Table 3. Variants of clinical progression of HIV with ongoing ART (n = 30)

Disease	HIV progression cases at various stages of ART									
	Total		1st year of ART		2nd year of ART		3rd year of ART		4th year of ART	
	abs.	rel., %	abs.	rel., %	abs.	rel., %	abs.	rel., %	abs.	rel., %
Tuberculosis	18	60	5	17	7	23	3	10	3	10
Herpetic infection	7	23	6	20	1	3	–	–	–	–
Candidiasis	3	10	3	10	–	–	–	–	–	–
Oral hairy leukoplakia	1	3	1	3	–	–	–	–	–	–
Cryptococcosis	1	3	1	3	–	–	–	–	–	–
Lymphoma	1	3	1	3	–	–	–	–	–	–
Total	31	103	17	57	8	27	3	10	3	10

100 cells/μL can be considered a borderline level at which it is not possible to predict immunological effectiveness of ART.

It should be noted here that virological effectiveness of treatment for at least the first year was the inclusion criterion, thus there no cases of VI of ART for that period of time to report. Virological effectiveness of ART in the second and third years of therapy was 92.7 %, which coincides with the data foreign authors report for a similar period of observation [11]. Same as in other studies, ART was VI mostly due to violation of regimen (80 % of cases in our study, 56 % — in the study by Klein et al. [12]). Only 8 % of patients that took part in our study and 9 % of the patients reported on in Klein's study had virus resistant to drugs. Initial drug-resistant HIV was detected in 5 of 34 study participants tested for such resistance. This justifies the need for testing for virus' drug resistance prior to start of ART, especially if the patient has had ART VI previously and/or was infected by a partner with drug-resistant HIV. The testing should allow choosing the optimal treatment regimen.

The share of "clinical progressors" with ART ongoing was quite high: 9 %. The majority of patients had tuberculosis in 1–4 years of therapy. 61 % of cases were associated with ART being II. We believe that TB could produce an adverse effect on the development of II of ART, because one-third (36.5 %) of study participants whose immune system did not respond to ART were ill with tuberculosis that developed either before the start of ART (22 % of patients with II of ART) or during ART (15 % of patients with II of ART), whereas the incidence of tuberculosis among patients for whom ART was effective equaled 12 % ($p < 0.001$). Other variants of clinical progression of HIV mainly occurred in the first year of ART. This is probably due to the general TB situation in Russia, which is adverse, and the threshold $CD4^+$ T-lymphocytes number that allows emergence of opportunistic infections. On the one hand, development of new secondary diseases when the number of $CD4^+$ grows is undesirable since it is associated with an increased risk of death and greater cost of treatment. On the other hand, such a development signals that the immune system is restoring with ART in the background. The average content of $CD4^+$ T-lymphocytes associated with clinical progression of HIV in the form of TB is 277 ± 194 cells/μL. This means that avoiding clinical progression requires starting ART early, when the content of $CD4^+$ is > 350 cells/μL, and prevention of secondary diseases (especially tuberculosis).

As for the risk of clinical progression of HIV infection when ART is II, the value we got, which is 6.232 times greater risk (95 % CI 3.106–12.51), is in the middle between the values other researchers reported [2, 3].

CONCLUSIONS

We have found that ART is II in 14.0–15.9 % of cases over one year and in 22 % of cases over 3 years. Of the 195 people that participated in the study from start to finish, 12 patients (6 %) showed ART II throughout all the years of treatment. It was noted that the number of cases of ART being II increases slightly from the first to the third year of treatment, and it is largely determined by the number of patients that had ART II after the first year of treatment. Adequate growth of $CD4^+$ T-lymphocytes after the first year of treatment requires the cells to grow their numbers by more than 100 cells/μL during the first year. This level can be used practically as an indicator of immunological effectiveness of ART for timely correction of treatment.

Virological effectiveness of ART was 92.7 % through two years. Ineffective viral load suppression was observed in 6.0 % of cases ad resulted from violation of regimen by patients. Only 0.6 % of cases were VI due to virus' drug resistance. Thus, proper attention to counseling aimed at forming a habit of sticking to the regimen can significantly improve virologic effectiveness of the treatment. The identified the original drug resistance of the virus (15 %) was associated with previous ART failures or infection from a partner with a drug-resistant virus. This fact necessitates testing this category of patients for HIV resistance to antiretroviral drugs before starting ART.

Clinical effectiveness of ART was 91 %. This result largely depends on the number participants for whom ART was II. TB was a leading disease clinical progression of HIV resulted in (61 % of cases). It is associated with a wide spread of values of content of CD4+ T-lymphocytes (25–616 cells/μL), which signals the need for an earlier start of ART, when CD4+ content is at > 350 cells/μL, and prevention of secondary diseases.

II of ART is a risk factor, the risk being progression of the disease with active ART in the background and death of the HIV-positive individual. This fact confirms the need for timely recognition of II and treatment regimen correction.

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DETECTING OCCULT HEPATITIS B WHEN TESTING DONATED BLOOD

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Individuals carrying occult (latent) hepatitis B pose epidemiological threat. Testing donated blood donors for surface antigen HBsAg (hepatitis B virus, HBV) only does not allow to assume the blood safe from the point of view of infections, which can result in post-transfusion transmission of infection. Lack of confidence here is due to the fact that the virus is present in the body even when HBsAg is negative. The study analyzes data of 61,155 blood donors of the Republican Blood Center (Kazan), collected in 2010–2014. The tests applied were those aimed at detecting HBsAg, anti-HBc-total, anti-HBc IgM (enzyme immunoassay), and determining DNA of the virus in the blood by polymerase chain reaction in "real time". It was found that donors with occult hepatitis B are identified each year, but their numbers decrease gradually. To prevent the spread of the virus it is recommended to add the anti-HBc-total test to the standard set of diagnostic tests.

Keywords: occult hepatitis B, latent hepatitis B, HBV infection, donor, blood, enzyme immunoassay, PCR diagnostics, HBsAg, anti-HBc-total, anti-HBc IgM

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ВЫЯВЛЕНИЕ ОККУЛЬТНОГО ГЕПАТИТА В ПРИ ТЕСТИРОВАНИИ ДОНОРСКОЙ КРОВИ

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Эпидемиологическую опасность представляют лица с оккультным (латентным) гепатитом B. Исследование крови доноров только на поверхностный антиген вируса (hepatitis B virus, HBV) HBsAg не может обеспечить полную инфекционную безопасность крови, следствием чего может быть посттрансфузионная передача инфекции. Это объясняется тем, что вирус присутствует в организме даже при отрицательном HBsAg. В работе проанализированы данные 61 155 доноров Республиканского центра крови (Казань), полученные в 2010–2014 гг. Анализировали результаты тестирования на HBsAg, anti-HBc-total, anti-HBc IgM (методом иммуноферментного анализа) и определения ДНК вируса в крови (методом полимеразной цепной реакции в «реальном времени»). Установлено, что доноры с оккультным гепатитом B выявляются ежегодно, хотя и отмечена тенденция к снижению их числа. Для предотвращения распространения вируса в популяции рекомендуется ввести в стандарт диагностики HBV-инфекции определение маркера anti-HBc-total.

Ключевые слова: оккультный гепатит B, латентный гепатит B, HBV-инфекция, донор, кровь, иммуноферментый анализ, ПЦР-диагностика, HBsAg, anti-HBc-total, anti-HBc IgM

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To avoid transmission of bloodborne infections from donor to recipient, it is imperative to ensure infectious safety of donated blood. Screening for hepatitis B (hepatitis B virus, HBV) helped to significantly reduce incidence of this infection [1]. However, despite availability of sensitive tests detecting HBV surface antigen (HbsAg), post-transfusion hepatitis B still occurs [2, 3]. This is due to the fact that the virus can lay dormant in liver tissue for a long time, and in some cases even in blood of HBsAg-negative patients [4, 5]. Therefore, occult (latent) hepatitis B is a risk factor in blood donation and organ transplantation [6]. There are documented cases of HBV DNA found in recipients that received Hbs-negative blood from donors who had occult HBV. Thus, there is a real threat of spread of latent hepatitis B and its further activation in human body when the immunity is suppressed [5].

The goal of our study was detection of latent HBV at the stage of donated blood testing.

METHODS

The study was conducted at the Republican Blood Center (RBC) of the city of Kazan. Data obtained from testing 61,155 donors in 2010–2014 were analyzed, namely: HBsAg tests, anti-HBc-total tests, anti-HBc IgM tests, HBV DNA tests.

HBsAg, anti-HBc-total and anti-HBc IgM were identified by ELISA using commercial reagents made by Vector-Best (Russia), sensitivity of 0,01 ME/ml. HBV DNA was detected by polymerase chain reaction in real time using COBAS S 201 (Roshe, Switzerland) device and a set of Sobas TaqScreen MPX Test reagents, v2.0 and CobasTaqScreen MPX ControlKit, v.2.0 (Roche).

RESULTS

HBsAg is the screening marker for HBV-infection. Detection of this marker when testing donated blood confirms presence of active infection. Individuals with HBsAg are dismissed as donors and receive applicable treatment. In 2010–2014, Kazan's RBC registered 6 to 25 cases of marker detection per year (Fig. 1), the trend is downward.

Anti-HBc-total tests returned positive in 3.5–6.9 % of cases a year, which means that blood from 400 to 900 donors could not be used (Fig. 2). When this marker is detected, the blood is subject to anti-HBc IgM. In 2010–2014, M-class immunoglobulins were detected in 2.5–9.9 % of donated blood samples, with signs of latent HBV in donors' bodies (anti-Hbc-total positive samples) (Fig. 3). Anti-HBc IgM is the viral activity market that signals the need for detailed medical examination of the person.

HBV DNA in blood serum is the main indicator of viral replication. Approximately 20 % of cases require solely DNA diagnostics to reveal latent HBV since no other tests are capable of detecting it [7]. At RBC, PCR is performed in real time to confirm absence of infection in donors that showed negative results for classical markers. This extra blood test also helps to detect active forms of HBV in cases of latent hepatitis B (Fig. 4).

DISCUSSION

In Russia, far from all medical institutions test donated blood for antibodies to hepatitis B (using anti-HBc-total or anti-HBc IgM tests), which hinders timely detection of latent forms of HBV. Our findings allow concluding that absence of HBsAg does not guarantee infectious safety of donated blood, since a positive anti-HBc-total test indicates previous exposure to the virus and a positive anti-HBc IgM test signals an active infection.

HBsAg-negative phase can show low level of virus DNA in the body: it is detected in liver and rarely in serum [8]. That is why this phase does not mean full recovery, since a relapse can occur any moment [9, 10]. Patients with latent HBV were found to maintain effective immunological response (anti-HBc total), which can lead to reactivation of HBV and HBsAg reappearance [11].

The complications of detection of occult hepatitis B arise from non-mandatory character of tests for markers of latent HBV

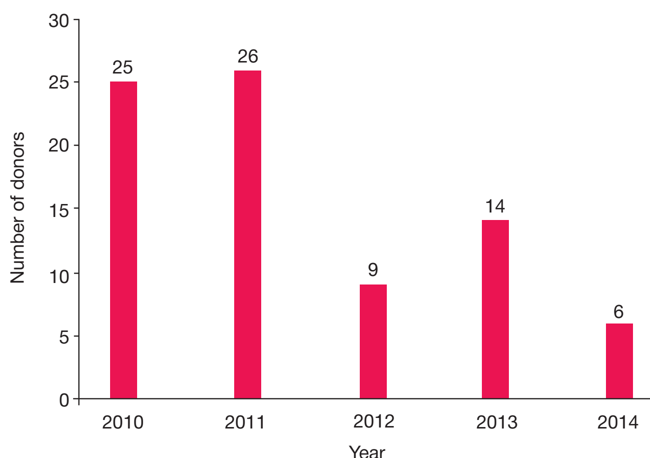

Fig. 1. Positive screening tests (HBsAg) statistics, 2010–2014

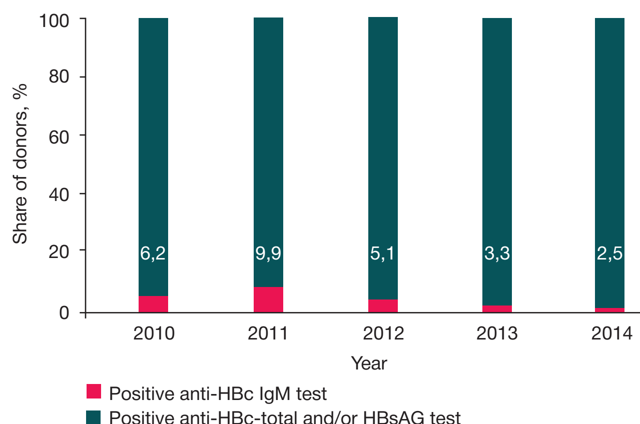

Fig. 2. Positive anti-HBc-total tests, 2010–2014

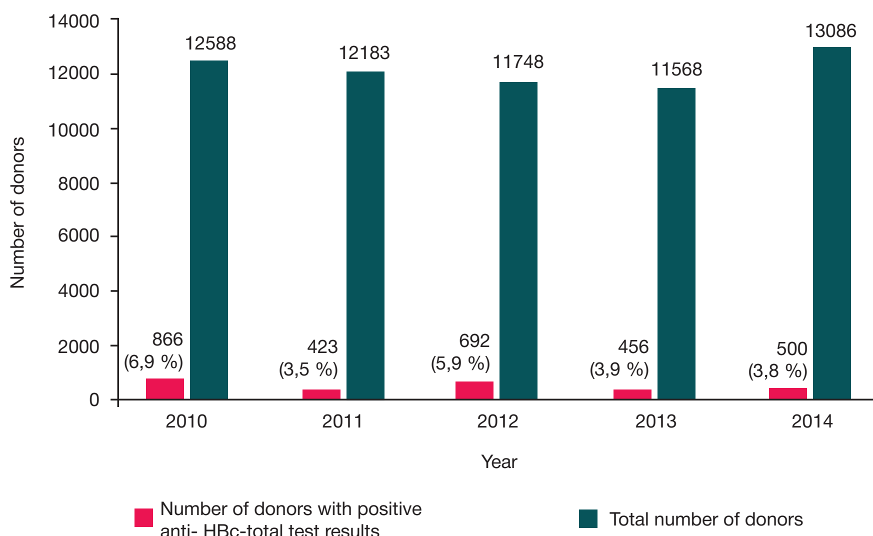

Fig. 3. Share of donors with M-class immunoglobulin in blood, donors with positive anti-HBc-total and/or HBsAg tests, 2010–2014

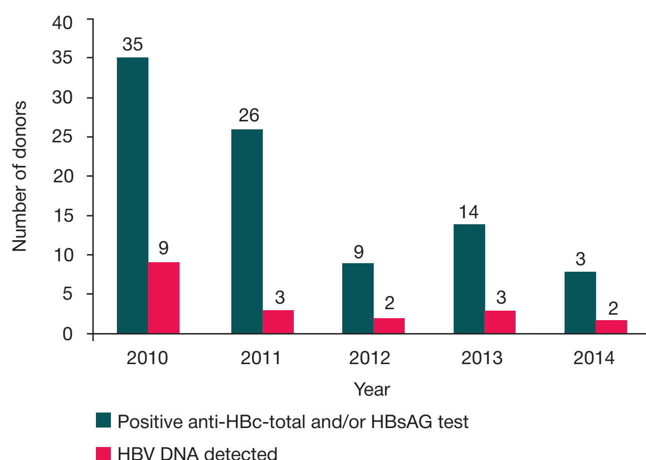

Fig. 4. Number of HBV DNA detection occurrences, donors with positive HBsAg and/or anti-HBc-total tests, 2010–2014

and further examination of the donor. Disease control center only receives information about donors with positive HBsAg tests. Timely implementation of preventive and anti-epidemic measures requires further medical examination and outpatient observation of individuals with anti-HBc-total and anti-HBc IgM in blood. Otherwise, use of blood from donors with occult hepatitis B increases the risk of transfusion transmission of HBV and its spread in the population [12, 13].

CONCLUSIONS

Donors with positive tests for HBV markers (HBsAg, anti-HBc-total, anti-HBc IgM, HBV DNA) are found every year. Introduction of anti-HBc-total test to the standard set of HBV diagnostic tests will increase the rate of detection of latent HBV [14, 15]. Setting up seamless workflow and cooperation between blood donation centers and other medical institutions will ensure early detection of active forms of infection and prevention of chronic process, and also help prevent infection of others.

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WOUND CARE WITH THE LEAF EXTRACT OF CECROPIN P1-PRODUCING TRANSGENIC KALANCHOE: HISTOLOGICAL FINDINGS

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Management of purulent wounds is a problem that requires particular attention: wounds are a common injury type for which suppurative complications are frequent, mortality rates are high and antimicrobial therapy may be ineffective due to the presence of drug-resistant bacteria in the wound. In this work we have studied the effectiveness of wound treatment with the leaf extract of transgenic *Kalanchoe pinnata* modified to produce antimicrobial peptide cecropin P1. Purulent wounds infected with *Staphylococcus aureus* were modeled in Wistar rats. Four groups of animals were formed, with 10 animals in each group. In all groups, the wounds were cleansed with 3 % hydrogen peroxide solution once a day; all groups except the controls received additional treatment. Group 2 received 10 % cefazolin solution, group 3 received kalanchoe juice, group 4 received the juice of cecropin P1-producing kalanchoe. Histologic stains of biopsy samples were performed after rats were sacrificed by anesthetic overdose on days 3, 10 and 14 after treatment onset. On day 3, wound dynamics was the same in all groups. On day 10 exudate was still observed in the controls; in group two exudation was almost finished and regeneration was about to begin; in groups 3 and 4 the wound defect was filled with granulation tissue. In spite of epidermal repair along the wound edges in groups 2 and 3, there still was some sloughing and granulation tissue was less mature than in group 4. We recommend conducting more extensive clinical research of the leaf extract of cecropin P1-containing transgenic *Kalanchoe pinnata*.

Keywords: wound healing, purulent wound, *Staphylococcus aureus*, *Kalanchoe pinnata*, cecropin P1, antimicrobial treatment

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ЛЕЧЕНИЕ РАН ЭКСТРАКТОМ ЛИСТЬЕВ ТРАНСГЕННОГО КАЛАНХОЭ С ЦЕКРОПИНОМ Р1 (ГИСТОЛОГИЧЕСКОЕ ИССЛЕДОВАНИЕ)

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Проблема лечения гнойных ран актуальна в хирургии в связи с распространенностью ран различной этиологии, частотой гнойных осложнений, высокой летальностью, появлением антибиотикорезистентных штаммов бактерий. В работе исследована эффективность фармакотерапии раневого процесса экстрактом листьев трансгенного каланхоэ перистого с антимикробным пептидом цекропином Р1. Гнойную рану моделировали на крысах линии Wistar с внесением в рану культуры *Staphylococcus aureus*. Сформировали 4 группы по 10 животных в каждой. Во всех группах раны обрабатывали ежедневно однократно 3 % раствором перекиси водорода и дополнительным препаратом, кроме группы 1 (контрольной). В группе 2 использовали 10 % раствор цефазолина, в группе 3 — сок каланхоэ, в группе 4 — сок каланхоэ с цекропином Р1. Гистологическое исследование раневых биоптатов производили на 3, 10 и 14 сутки с начала лечения после выведения крыс из эксперимента путем передозировки наркоза. Результаты лечения через 3 сут были схожими во всех группах. Через 10 сут для ран крыс контрольной группы была отмечена незавершенность фазы экссудации, группы 2 — переход фазы экссудации в фазу регенерации, а групп 3 и 4 — покрытие грануляционной тканью. Несмотря на восстановление эпидермиса по краям ран в группах 2 и 3, кое-где сохранялся струп, а грануляционная ткань была менее зрелой, чем в группе 4. Результаты позволяют рекомендовать экстракт листьев трансгенного каланхоэ перистого с цекропином Р1 для широкого клинического изучения.

Ключевые слова: раневой процесс, гнойная рана, *Staphylococcus aureus*, каланхоэ перистое, *Kalanchoe pinnata*, цекропин Р1, антимикробная терапия

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Treatment of purulent wounds of various origins is complicated by frequent suppurative complications and associated with high fatality rates entailing considerable expenses [1, 2]. According to some authors, suppurative complications account for 30–35 % of all surgical conditions, causing death in 25 % of cases [3–5].

There are a lot of approaches to treating purulent wounds [6–10]; new methods are also being continuously developed and introduced into clinical routine. Among them are hyperbaric oxygen therapy, laser therapy, magnet therapy, wound treatment in the aseptic environment, etc. [11–15]. But the most common method relies on the use of dressings, since they are available, easy to use and cheap [16–18].

It should be noted that overuse or misuse of antibacterial drugs promotes antibiotic resistance in bacteria impeding treatment of complex chronic diseases, such as venous leg ulcers in diabetic patients who have to undergo a long-term antibacterial therapy [19, 20]. Promising alternatives to traditional antibiotics are antimicrobial peptides and biostimulators that promote healing, such as kalanchoe.

Many plants of the genus *Kalanchoe* are medicinal herbs: their juice is used to treat burns, dermal wounds, and ulcers; they can be used as biostimulators after skin grafting. Kalanchoe juice is rich in flavonoids, such as bufadienolides and lectins known to trigger mitosis in lymphocytes, vitamins, organic acids, polysaccharides, antioxidants, and micronutrients [21, 22]. So far, transgenic plants — “bioreactors” for producing active pharmaceutical ingredients — have been engineered based on *K. daigremontiana* [23, 24], *K. laciniata* [25] and *K. blossfeldiana* [26]. Of them all, *K. pinnata* has the most substantial pharmacological potential. In 2012 a new method was developed to obtain transgenic *K. pinnata* plants in which the gene of cecropin P1 is expressed [27].

Cecropin P1 is a secretory factor of a large roundworm of pigs, *Ascaris suum*; it belongs to the group of linear α-helical peptides that do not contain cysteine [28]. Unlike insect cecropins, porcine cecropin P1 consists of a long positively charged α-helix that carries a large number of amino acid residues. In the experiments *in vitro*, cecropin P1 has been shown to be highly active against gram-positive and gram-negative pathogenic bacteria [29], fungi [30] and some tumor cells [31], but its antimicrobial activity *in vivo* has not been reported so far.

In light of the above, our work aimed to assess the effect of the leaf extract of transgenic cecropin P1-producing *K. pinnata* on purulent wounds infected with *Staphylococcus aureus* in the rat model.

METHODS

The experiment was carried out in Wistar rats (age of 4 months, weight of 200–220 g) that had been quarantined prior to the experiment in the animal facility of the Research Institute for Environmental Medicine of Kursk State Medical University. Only healthy animals were chosen for the experiment. The animals were housed in a standard biologically clean room at 22–24 °C under 12/12 light cycle. All rats received pellet food and filtered tap water. Treatments were conducted in the afternoon at a fixed time. The rats were anaesthetized with intraperitoneal injections of the chloral hydrate aqueous solution, 300 mg/kg body weight, and sacrificed with its overdose. The experiment was conducted in compliance with the principles of the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes (Strasburg, 1986).

The animals were stratified by body weight and the type of treatment applied. The rats with a modeled purulent wound infected with *S. aureus* were distributed into the following groups: group 1 (controls) consisted of animals treated with 3 % hydrogen peroxide (n = 10); group 2 included animals treated with 3 % hydrogen peroxide and 10 % cefazolin solution (n = 10); group 3 included animals treated with 3 % hydrogen peroxide and kalanchoe juice (n = 10); group 4 consisted of animals treated with 3 % hydrogen peroxide and transgenic kalanchoe juice with cecropin P1 (n = 10). Wounds were modeled on the anaesthetized animals under sterile conditions. A 20 × 20 mm area on the back was shaved to remove hair and treated with an aseptic followed by the excision of the skin and subcutaneous tissue. Then 1 ml of 10^6 CFU/ml *S. aureus* 592 solution (a 24-h old culture) was introduced into the wound. To provide uniform treatment conditions, prevent the wound from deformation, drying, contamination or animal bites, a piece of gauze dressing was sewn onto the skin to cover the wound. In 48 h all animals showed typical signs of inflammation and suppuration. After sutures were removed, the dressing was taken off, puss was drained and wound care was performed once a day for 14 days in a row.

Biopsy was performed on days 3, 10 and 14 after treatment onset; the animals were sacrificed in threes. Soft tissue samples were excised from the wound bed and the adjacent edges using the razor. The samples were immediately fixed in 10 % neutral formalin solution, dehydrated through an ascending series of alcohols and embedded into paraffin according to the standard protocol. Paraffin slices were stained with hematoxylin and eosin.

Microscopy and microimaging were performed using the optical system consisting of Leica CM E microscope (Leica Microsystems, Germany) and Micromed DCM-510 SCOPE digital camera (Nablyudatelnie pribory, Russia) at magnifications of ×40, ×100, ×200 and ×400; images were captured using Future Win Joe software (Future Optics, China) supplied with the digital camera. In the course of the histological analysis, we assessed inflammation intensity, the onset of granulation, epithelialization at wound edges, and quality of the new epithelium. A cell profile of the tissue adjacent to the wound edges or of the newly formed tissue at later healing stages was also prepared. Fibrous tissue was differentiated from other cells karyologically. The proportion of various type cells was calculated after counting 100 cells in ≥ 10 non-overlapping fields of view.

Statistical analysis was performed using Microsoft Excel 10.0. Mean value (M) and standard error of mean (m) were computed for all parameters. The two-sample t-test with unequal variances was applied to compare the groups and establish differences between them. Differences were considered significant at $p < 0.05$

RESULTS

Treatment outcome in group 1 (controls)

Day 3

Purulent wounds showed signs of acute suppurative inflammation. The wound surface was covered with fibrinous and leukocyte detritus; underneath, degrading leukocytes were accumulating and hemorrhagic areas were observed. Tissues adjacent to the wound were edematous showing signs of leukocyte infiltration. Edema and infiltration were observed in deeper layers, down to the muscle tissue. The epithelium at the

wound edges was thickened and disorganized. In the dermis, collagen fibers were swollen and blood vessels were dilated and plethoric (Fig. 1, A).

Day 10

The wounds were filled with a purulent necrotic mass; the epidermis at the wound edges was thickened. Macrophages and mast cells were observed in the dermis which showed conspicuous leukocyte infiltration (see Table). Interstitial edema had spread into deeper dermal layers down to the muscle fibers (Fig. 1, B).

Day 14

Deep leukocyte infiltration was still present; the wounds were filled with necrotic tissue. The dermis was edematous at the wound edges, and the epidermis was thinned. Edema and infiltration persisted in the underlying muscle tissue. Granulation tissue started to develop in the wound bed; occasional microabscesses were observed filled with leukocytes (Fig. 1, C).

Treatment outcome in group 2 (10 % cefazolin solution)

Day 3

Histology revealed signs of acute suppurative inflammation. The wounds were filled with necrotic tissue; multiple leukocyte and fewer neutrophil infiltrates were observed. A large number of macrophages were spotted in a field of view (Fig. 1, D).

Day 10

Connective tissue was actively growing in the dermis to form the organized structure; fibroblastic cells and macrophages were abundant (see Table). Epithelial cells were vigorously proliferating and differentiating. (Fig. 1, E).

Day 14

Areas of the new epidermis with clearly differentiated layers were noticed, but its thickness exceeded that of the intact skin. Granulation tissue was mature. A few inflammatory microfoci were spotted in deeper dermal layers. On the whole, the wound granulated actively; granulation tissue was subsequently replaced by fibrous tissue. The skin defect was covered with multiple collagen fibers running in different directions (Fig. 1, F).

Treatment outcome in group 3 (kalanchoe juice)

Day 3

We observed a morphological pattern similar to that in the controls and group 2. Mast cells were actively involved in the reparative process indicated by the increased number of total cells near the wound. The wound retained residual purulent exudate and necrotic tissue. (Fig. 2, A).

Day 10

The epidermis at the edges started to advance to the wound bed where foci of granulation tissue had already appeared. Single collagen fibers were arranged chaotically surrounded predominantly by fibroblasts and macrophages (Table, Fig. 2, B).

Day 14

In some samples, complete epithelialization was observed. Variably mature granulation was observed in the derma. Collagen fibers were surrounded by fibroblasts and ran parallel to the skin surface (Fig. 2, C).

Treatment outcome in group 4 (juice of transgenic kalanchoe producing cecropin P1)

Day 3

The wounds were filled with necrotic tissue. The samples contained a lot of neutrophils. Marked edema and dilated capillaries were observed in the derma (Fig. 2, D).

Day 10

Regeneration was accompanied by a localized inflammatory response induced by the arrival of neutrophils at the wound site. Inflammatory infiltrates were polymorphic. The wound bed was granulating. Vigorous angiogenesis improved tissue vascularization in the area surrounding the wound; edema diminished, inflammatory infiltration decreased (Fig. 2, E).

Day 14

The wound was filled with multiple collagen fibers surrounded by fibroblasts. Fibers were arranged chaotically, though horizontal orientation prevailed. The epithelium was advancing growing over the granulation tissue. It was thicker than the intact skin (Fig. 2, F).

Comparison of treatment outcomes

Histological analysis conducted on day 3 of the experiment did not reveal any significant differences in treatment outcomes in different groups. However, on day 10 the situation was different. The exudative phase was still unfinished in the control group. In group 2 the exudative phase was giving way to the remodeling phase. In groups 3 and 4 the wounds were granulating.

On day 14 the number of fibroblasts significantly exceeded the number of granulocytes and macrophages in groups 3 and 4 (see Table) indicating an active regenerative process. Regeneration was the most successful in group 4 (transgenic kalanchoe with cecropin P1). By the end of the experiment the wounds in this group had been fully covered with the new epidermis.

DISCUSSION

Since 2007, a number of authors have described the process of obtaining a transgenic *K. pinnata* able to express the cecropin P1 gene and accumulate this peptide in the cytoplasm [30]. Accumulation of cecropin P1 and its effect on phytopathogens and traditional bacterial cultures have been assessed *in vitro*. However, neither of those works contain any information about the therapeutic effect of transgenic kalanchoe juice in the treatment of infections in animas. Still, healing, immunomodulatory and remodeling properties of *K. pinnata* [32] may enhance the antibacterial effect of cecropin P1 in the experiments with the transgenic plant *in vivo*.

Our study demonstrates that kalanchoe juice expedites transition from the first stage of the inflammatory process to remodeling. In comparison with the controls, kalanchoe

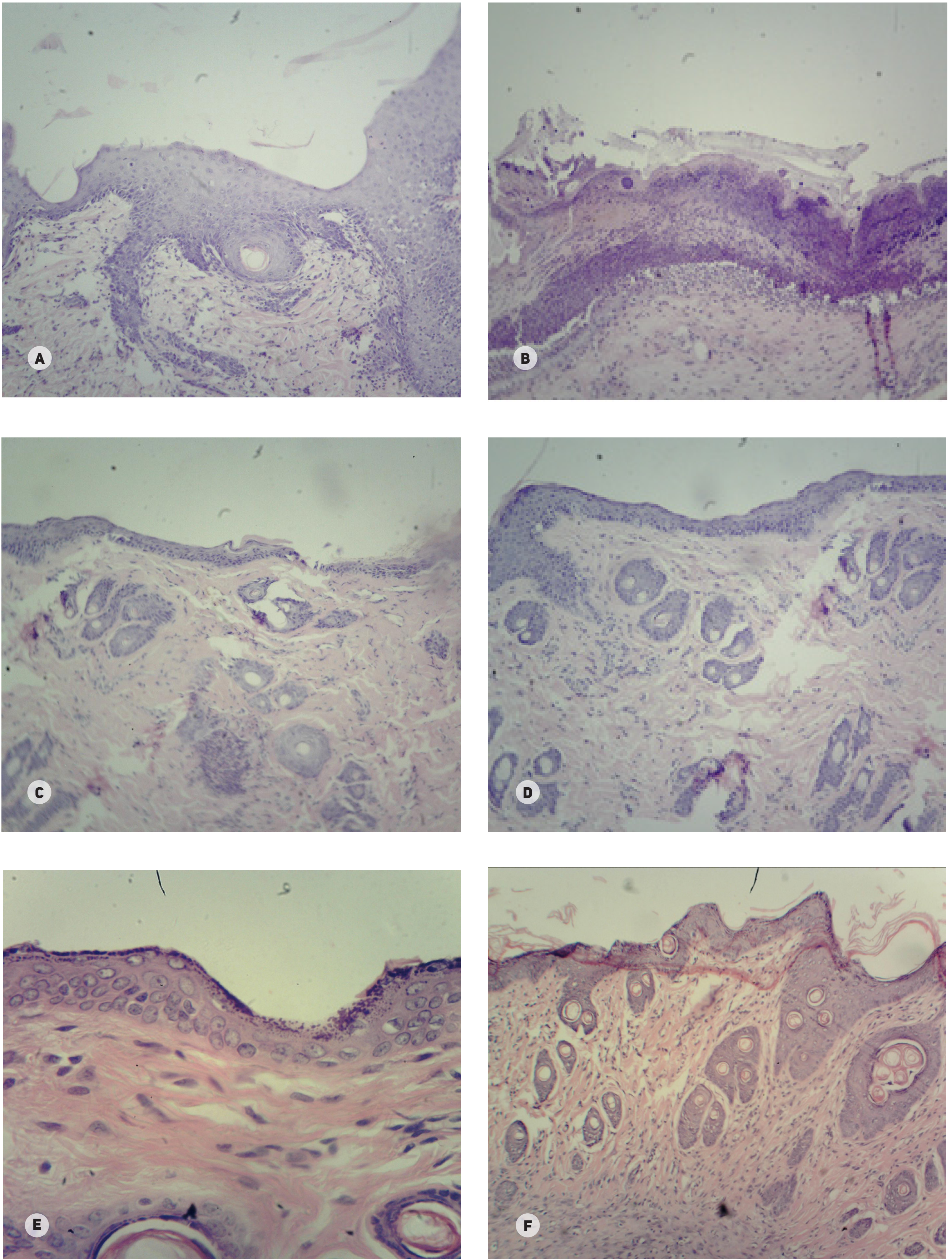

Fig. 1. Histological study of wounds modeled in rats. **(A–C)** Histological slices of animals in the control group (group 1) sacrificed at different stages of the experiment: **(A)** on day 3; **(B)** on day 10; **(C)** on day 14. **(D–F)** Histological slices of animals in group 2 (additional treatment with 10 % cefazolin) sacrificed at different stages of the experiment: **(D)** on day 3; **(E)** on day 10; **(F)** on day 14. Hematoxylin and eosin staining, ×280

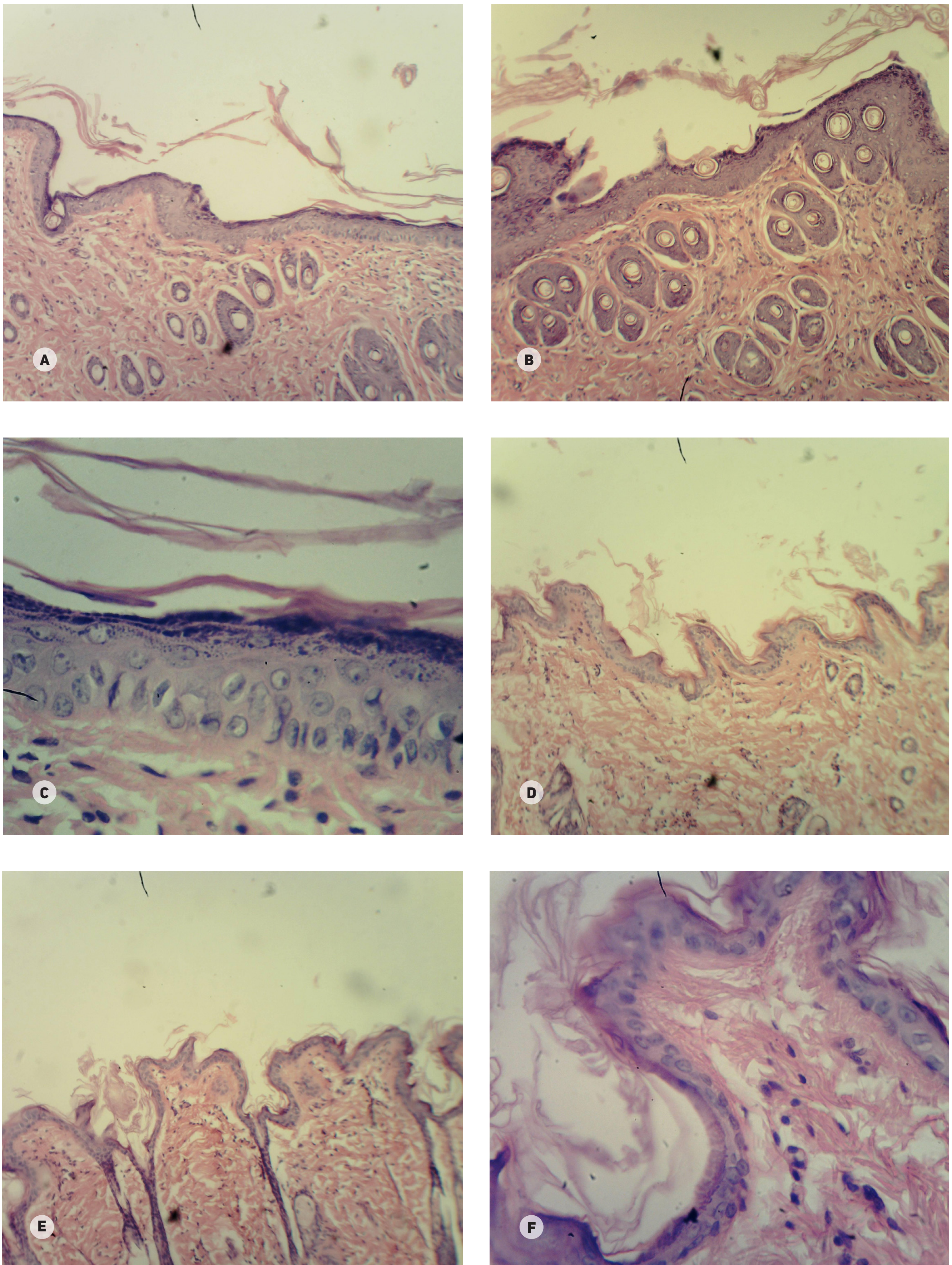

Fig. 2. Histological study of wounds modeled in rats. **(A–C)** Histological slices of animals in group 3 (additional treatment with kalanchoe juice) sacrificed at different stages of the experiment: **(A)** on day 3; **(B)** on day 10; **(C)** on day 14. **(D–F)** Histological slices of animals in group 4 (additional treatment with kalanchoe juice containing antimicrobial peptide cecropin P1) sacrificed at different stages of the experiment: **(D)** on day 3; **(E)** on day 10; **(F)** on day 14. Hematoxylin and eosin staining, ×280

Proportion of various cells in the modeled wounds with regard to the treatment type and the day of sacrifice

Group	Day of sacrifice											
	3				10				14			
	Fibro-blasts	Macro-phages	Granulo-cytes	Lympho-cytes	Fibro-blasts	Macro-phages	Granulo-cytes	Lympho-cytes	Fibro-blasts	Macro-phages	Granulo-cytes	Lympho-cytes
1 (controls)	14,7 ± 0,7	12,9± 0,3	49,9 ± 1,8	22,5 ± 0,6	17,0 ± 0,4	13,3 ± 0,2	48,0 ± 2,8	21,7 ± 0,3	24,9 ± 0,5	38,7 ± 1,7	15,6 ± 0,8	20,8 ± 0,3
2 (10 % cefalozin solution)	9,5 ± 0,2	59,3 ± 2,1	7,9 ± 0,2	23,3 ± 0,2	15,2 ± 0,1	62,4 ± 1,3	7,5 ± 0,4	14,9 ± 0,6	31,9 ± 0,7	51,7 ± 2,6	8,4 ± 0,1	8,0 ± 0,1
3 (kalanchoe juice)	12,9 ± 0,6	37,1 ± 0,7	30,3 ± 1,6	19,7 ± 0,4	17,7 ± 0,2	38,9 ± 1,6	15,6 ± 0,2	27,8 ± 0,4	24,0 ± 0,6	47,3 ± 2,5	14,4 ± 0,2	14,3 ± 0,2
4 (kalanchoe juice + cecropin P1)	12,1 ± 0,2	21,4 ± 0,4	44,0 ± 1,2	22,5 ± 0,1	20,5 ± 1,0	23,9 ± 0,4	24,2 ± 1,1	31,4 ± 2,6	30,1 ± 1,2	26,4 ± 1,6	18,4 ± 1,1	25,1 ± 1,2

treatment promoted faster elimination of edema and debridement of necrotic tissue, stimulated granulation and epithelialization. The polymorphic cell profile and the presence of variably mature fibroblastic cells indicate a stimulating effect of the transgenic kalanchoe on the proliferative and functional activity of granulation tissue. The therapeutic effect of transgenic kalanchoe juice is very marked at the last stage of wound healing in comparison with the controls. A combination treatment with 1 % cefazolin and kalanchoe juice was more effective than the treatment received by the controls. Still it was less effective than in the group treated with transgenic kalanchoe: in spite of complete regeneration of the epidermis at the wound edges in groups 2 and 3, sloughing was still observed, and the presence of various cell types in the granulation tissue indicated that it was less mature in comparison with group 4. Full epidermal closure in group 4 and integumentary structures indicated faster regeneration and complete skin restoration due to a more active migration and proliferation of endotheliocytes and vigorous angiogenesis.

Surprisingly, transgenic kalanchoe juice produced a stronger inhibitory effect on the growth of *S. aureus in vivo* than we had expected knowing about its antibacterial activity demonstrated in some experiments in bacterial cultures *in vitro* [30]. This might be due to the combined antimicrobial effects of P1 cecropin and endogenous bufadienolides of *K. pinnata*. Besides, the rate of pathogen elimination from the wound could be determined to a great extent by hemagglutination of *K. pinnata* lectins that have an immunostimulatory effect [33]. Similar to concanavalin and phytohemagglutinin, these lectins can promote proliferation of lymphocytes, including those that recognize specific pathogen antigens accumulating near the infection site. Coupled with cecropin P1-induced suppression of bacterial functions and the antimicrobial factors of the juice, lectins can promote healing and stimulate production of antigens. Also, pathogen elimination from the wound can be expedited by vascularization and scar tissue remodeling induced by some kalanchoe juice components, yet unidentified. This can stimulate transport of immune system cells and soluble factors to the infection site promoting better healing.

CONCLUSIONS

We have demonstrated a strong therapeutic effect of transgenic kalanchoe juice (with cecropin P1) on purulent wounds infected with *S. aureus* in rats. The obtained results allow us to recommend the studied substance for use in the clinical setting. Transgenic kalanchoe juice is a promising agent for treating varicose leg ulcers in patients with diabetes. Their immunity is compromised and the ulcers often harbor mixed infections; however, long-term therapy is complicated by drug resistance that bacteria develop in the course of treatment.

In cases of external bacterial infections, the use of transgenic kalanchoe juice instead of the extracted antimicrobial peptide will reduce purification expenses and enhance the therapeutical effect of cecropin P1. Thus, *K. pinnata* may be recommended as a promising, cost-effective and available “bioreactor” for obtaining peptide- or protein-derived antimicrobial substances, including alpha-lytic protease, lysostaphin and hirudin.

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KEY PERFORMANCE INDICATORS FOR HEALTHCARE RESEARCH ORGANIZATIONS BETWEEN 2011 AND 2015

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In this work we identify 16 key indicators to evaluate the performance of healthcare research organizations. These indicators comprehensively characterize such aspects of performance as research output and relevance, human resource development, integration into the international scientific community, distribution of scientific knowledge, promotion of the prestige of science, and resource provision. Below, we review the existing classification of medical research institutions and their key features. We present the results of the comprehensive performance evaluation of healthcare research organizations. We demonstrate the significance of the proposed indicators that accurately reflect the output and relevance of scientific research and stress that indicators currently used for performance evaluation are insufficient. We also emphasize the need for a systemic approach to personnel capacity assessment and confirm the importance of additional evaluation criteria that amount to 37.5 % of all key indicators.

Keywords: research organization performance, key performance indicators, healthcare research organization, state-funded research organization

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КЛЮЧЕВЫЕ ПОКАЗАТЕЛИ РЕЗУЛЬТАТИВНОСТИ ДЕЯТЕЛЬНОСТИ НАУЧНЫХ ОРГАНИЗАЦИЙ В СФЕРЕ ЗДРАВООХРАНЕНИЯ ЗА 2011–2015 ГГ.

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Определены 16 ключевых критериев результативности деятельности научных организаций в сфере здравоохранения, характеризующих результативность и востребованность научных исследований, развитие кадрового потенциала, интеграцию в мировое научное пространство, распространение научных знаний и повышение престижа науки, ресурсное обеспечение деятельности организаций. В рамках классификационного подхода рассмотрены медицинские научные учреждения, показаны их особенности. Представлена системная оценка результативности деятельности научных организаций в сфере здравоохранения. Продемонстрирована высокая значимость показателей, отражающих результативность и востребованность научных исследований. Отмечены недостаточность применяемых параметров и отсутствие системного подхода к анализу кадрового потенциала. Доказана важная роль дополнительных критериев оценки, составивших 37,5 % общей доли ключевых показателей.

Ключевые слова: результативность деятельности научной организации, ключевые показатели результативности, научная организация в сфере здравоохранения, государственная научная организация

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Evaluating the performance of healthcare research institutions is exceptionally significant and of vital importance for the further development of Russia's research potential. The importance of this analysis was dictated by the need to increase efficiency amidst modernization of the public sector of science [1, 2]. On the other hand, modern theoretical and practical developments and tools used to assess the output of medical research institutions often do not facilitate comprehensive monitoring of their activities.

The peculiarity of the Russian methodological approach to analysis of the performance of healthcare research organizations is that a large number of criteria are used, unlike what is obtainable in a number of Western countries [3, 4]. A typical evaluation method applied in Russia [2] is based predominantly on quantitative rather than qualitative indicators. Additional criteria are used for systemic monitoring, including for institutions with bed space facilities. Since the outcome of an analysis directly determines financing decisions, identification of key criteria is particularly important.

The activities of medical research institutions have characteristic specificity and important differences from the activities of other research institutions [5, 6]. Performance

indicators approved for healthcare research institutions include monitoring of the following areas:

- output and relevance of scientific research;
- human resource development;
- integration into the international scientific community, dissemination of scientific knowledge and promotion of the prestige of science;
- resource support for the activities of the research institution.

METHODS

Performance indicators for 55 research institutions subordinated to the Russian Ministry of Health for the period 2011–2015 were investigated.

In accordance with the accepted classification, the above research institutions subordinated to the Russian Health Ministry (n = 55) were divided into three groups: those with bed space facilities (n = 41), those without bed space facilities (n = 4), expert and socially important organizations (n = 10).

Statistical analysis was performed using software package Statistica 10.0. The Kolmogorov–Smirnov test by Lilliefors was used to test statistical hypotheses about type of distribution. It was found that in all cases, the data were not subject to the normal law of distribution; so nonparametric tests were used for statistical analysis.

Kruskal–Wallis test and the median test were used to determine the key performance indicators for healthcare research organizations. Additionally, the years 2011 and 2015 were compared in terms of Mann–Whitney U test and it was found that these figures change sufficiently enough.

Correlation analysis was performed using the Spearman's rank correlation coefficient. Indicators that change over time were included in the correlation analysis with background indicators for 2011, since it is the starting point of reference. P-value < 0.05 was taken as the level of statistical significance.

RESULTS

Most research institutions (74.5 %) under the Russian Ministry of Health have bed space facilities. However, this does not simplify the assessment procedure — you cannot apply indicators characterizing healthcare delivery to the other research organizations. In this regard, additional criteria are applied in analyzing the performance of research institutions with bed space facilities.

Statistical analysis of the 5-year dynamics (2011–2015) of performance criteria uncovered 16 key performance indicators ($p < 0.05$). The structure of the criteria earmarked and their proportion in the total number of parameters are shown in Fig. 1 and 2.

For the period 2011–2015, the following key indicators were identified.

Key performance indicators and relevance of scientific research:

1. Cumulative impact factor of journals where the articles of the organization are published. It should be noted that this indicator was found to have a moderate correlation with the number of *Doctors of Sciences* (the highest academic degree in Russia and many other post-Soviet states obtained after obtaining a PhD degree) ($r_s = 0.455$, $p < 0.01$), with intramural ($r_s = 0.449$, $p < 0.01$) and extramural ($r_s = 0.411$, $p < 0.01$) current expenditure on scientific research and development, and with the number of articles of the organization published in journals indexed in Web of Science ($r_s = 0.406$, $p < 0.01$).

2. Number of used intellectual property transferred under a license agreement. It was found that this indicator has moderate correlation with intramural current expenditure on research and development, including expenditure on exploratory research ($r_s = 0.475$, $p < 0.01$).

3. Number of used intellectual property contributed into the authorized capital.

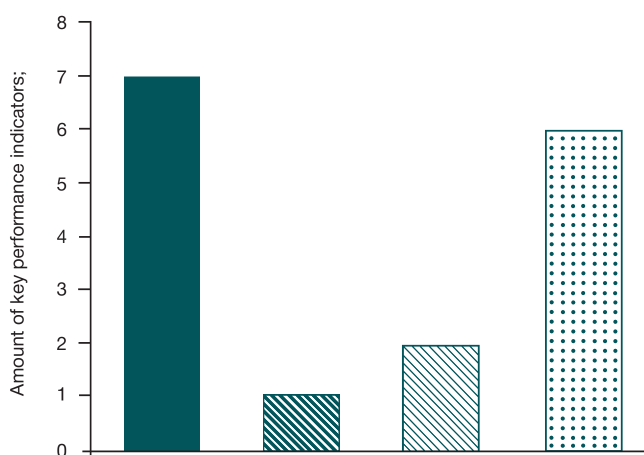

Fig. 1. Structure of key performance indicators for healthcare research institutions

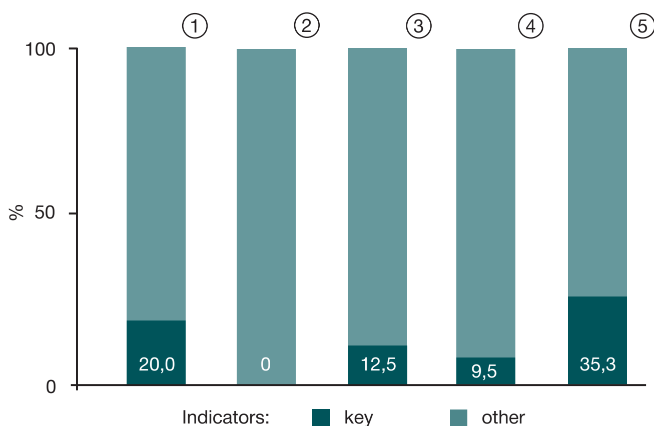

Fig. 2. Proportion of key performance indicators for healthcare research institutions in the total number of indicators in each field

4. Number of small innovative enterprises created with the participation of that institution.

5. Cumulative average staff number at small innovative enterprises.

6. Cumulative income at small innovative enterprises.

7. Financial impact of the research institution by income sources. This indicator was found to have a weak relationship with intramural current expenditure on research and development ($r_s = 0.302$, $p < 0.05$), including a positive correlation with basic research ($r_s = 0.351$, $p < 0.01$) and negative correlation with exploratory research ($r_s = –0.324$, $p < 0.05$).

Key indicator: integration into the global research community, dissemination of scientific knowledge and enhancement of prestige of science

The number of traffic (visits) to official sites and/or web pages of the institution on the Internet is defined by a single key indicator. The criterion was found to have a moderate correlation with the number of positive and neutral mentions of the institution in the federal media ($r_s = 0.357$, $p < 0.01$), including in online publications ($r_s = 0.325$, $p < 0.05$), and with the number of publications of the organization in journals indexed in the Russian Science Citation Index ($r_s = 0.339$, $p < 0.05$).

Key indicators: resource support for the research institution:

1. Expenditure on fixed assets and intangible assets, including buildings and structures, machinery and equipment. The indicator was found to have a moderate correlation with financial performance of research institution by type of work performed and services rendered, including production goods, works and services ($r_s = 0.491$, $p < 0.01$).

2. Intramural current expenditure on basic research. This indicator correlated with the financial performance of research institution by type of work performed and services rendered: by number of research and developments ($r_s = 0.548$, $p < 0.01$); with number of employees engaged in research and development ($r_s = 0.516$, $p < 0.01$), including researchers ($r_s = 0.418$, $p < 0.01$), among whom are PhD holders ($r_s = 0.405$, $p < 0.01$) and *Doctors of Sciences* ($r_s = 0.368$, $p < 0.01$) aged not above 39 years ($r_s = 0.505$, $p < 0.01$).

All the key indicators of resource support for a research institution were interconnected with the financial performance of the research institution by type of work performed, by services rendered and by income sources.

Key additional performance indicators for a research institution:

1. Number of research critical technologies from the list approved by the Scientific Council of the Russian Ministry of Health. This indicator correlated with the number of publications in journals indexed in Scopus ($r_s = 0.367$, $p < 0.01$), and with the number of innovative medical technologies used at the institution and approved by the Scientific Council of the Russian Ministry of Health ($r_s = 0.356$, $p < 0.01$). The indicator was found to be weakly correlated with the number of positive and neutral mentions of the organization in the federal media ($r_s = 0.295$, $p < 0.05$), including in the federal print media, television and radio ($r_s = 0.291$, $p < 0.05$), and with the number of researchers sent to work in leading Russian and international research and educational organizations ($r_s = 0.268$, $p < 0.05$).

2. The proportion of highly skilled medical doctors out of the total number of medical doctors. Curiously, this indicator was found to have a negative correlation with the total number of scientific, design and technological works ($r_s = –0.273$, $p < 0.05$), with the number of positive and neutral mentions of the organization in the media ($r_s = –0.311$, $p < 0.05$), with financial performance of the research institution by type of work performed and services rendered (by research and development) ($r_s = –0.308$, $p < 0.05$), and with the number of prepared draft healthcare delivery standards and procedures, clinical practice guidelines and other regulations ($r_s = –0.276$, $p < 0.05$).

3. Number of innovative medical technologies used at the institution and approved by the Scientific Council of the Russian Ministry of Health. This indicator correlated with the number of publications of the organization in journals indexed in Scopus ($r_s = 0.367$, $p < 0.01$), with the number of intellectual property created ($r_s = 0.406$, $p < 0.01$), including those with state registration and/or legal protection in Russia ($r_s = 0.392$, $p < 0.01$), and with the percentage of residents of other regions that received high-tech health care (HHC) at that institution ($r_s = 0.360$, $p < 0.01$).

4. Federal nature of the institution: the percentage of people from other regions who have received specialized medical care in that institution. This indicator moderately correlated with the number of people from other regions who received HHC ($r_s = 0.474$, $p < 0.01$), and with the number of Federal subjects of Russia, whose residents were treated in the reporting year ($r_s = 0.437$, $p < 0.01$).

5. Percentage of medical care expenses under mandatory health insurance (percentage of the total cost). This indicator correlated with the number of intellectual property created ($r_s = 0.350$, $p < 0.01$), including those with state registration and/or legal protection in Russia ($r_s = 0.459$, $p < 0.01$), with financial performance of the research institution by income sources ($r_s = 0.369$, $p < 0.01$), with intramural current expenditure on basic research ($r_s = 0.417$, $p < 0.01$).

6. Number of prepared draft healthcare delivery standards, draft healthcare delivery procedures, clinical practice guidelines (treatment protocols) and other regulations. This indicator was found to be correlated with the number of publications of the organization in journals indexed in Scopus ($r_s = 0.384$, $p < 0.01$), with the financial performance of the research organization by type of work performed and services rendered, including educational services ($r_s = 0.394$, $p < 0.01$), specialist training under continuing professional education programs on unique technologies along the profile of that research institution ($r_s = 0.449$, $p < 0.01$), and with the percentage of residents of other regions who received HHC ($r_s = 0.358$, $p < 0.01$).

DISCUSSION

Analysis of performance indicators for research institutions subordinated to the Russian Health Ministry identified 16 key criteria, most (43.75 %) of which characterize the output and relevance of scientific research.

Scientometric analysis, which is of particular importance for systemic evaluation [7–9], revealed that among the criteria analyzed (number of publications in journals indexed in the Russian Science Citation Index, Web of Science and Scopus, total number of citations of publications of the institution, etc.), the cumulative impact factor of journals where the articles of the organization are published is a key indicator. This is probably due to a more careful selection of journals — since publications in top-ranking journals, including foreign journals, increase not only the citation of authors, but also the interest of foreign colleagues in Russian research and future collaboration.

The innovative development model of Russian science involves closer relationship with the economic sector [10]. Against this background, a special role belongs to small innovative enterprises and key indicators reflecting their activities. However, over the studied period — 2011–2015 — medical research institutions showed virtually no interest in commercialization of their intellectual products. It was found that small innovative enterprises created only 14.5 % of organizations with bed space facilities.

The standard human resource evaluation for healthcare research organizations includes four quantitative indicators, none of which is a key indicator. The absence of qualitative factors and the different importance of investigated parameters create obstacles to a full-fledged analysis. According to a number of researchers [5, 11], an effective human resource evaluation requires a systemic approach, which includes monitoring of professional competence, learning ability, conditions and wages, and many other indicators.

Since the output of institutions depends on the research performed and other works related to the main activities of the institutions, as well as on conformity of the state of national science to modern world standards [12–15], there is need to develop quality criteria for a particular area.

During the period under consideration — 2011–2015 — despite a decline in the number of mentions of research organizations in the media, a sharp increase in visits to the official websites of medical research institutions was revealed. Presumably, this is due to increased online activity by interested persons.

The use of additional performance criteria for evaluation of healthcare research organizations has proved its worth — they accounted for 37.5 % of the total number of key indicators. It is curious to note that the criterion reflecting the number of used scientific critical technologies from the list approved by the Scientific Council of the Russian Health Ministry correlates with the number of publications of the organization in journals indexed in Scopus and with the number of researchers sent to work in leading Russian and international research and educational organizations.

CONCLUSIONS

In assessing the performance of healthcare research organizations in the period 2011–2015, 16 key performance indicators were identified, among which are especially significant criteria, reflecting the output and relevance of scientific research.

During the evaluation, the additional criteria were found to be relevant. Data obtained clearly show that the used research critical and innovative medical technologies from the list approved by the Scientific Council of the Russian Health Ministry, prepared draft healthcare delivery standards, procedures and clinical practice guidelines are crucially important for further development of the scientific potential of institutions.

For institutions with bed space facilities, the proportion of highly qualified medical doctors, the percentage of people from other regions who have received specialized medical care, and the percentage of medical care expenses under mandatory health insurance served as additional key indicators.

It was shown that the existing standard indicators for human resource analysis are insufficient and that there is need to come up not only with quantitative but also with qualitative criteria.

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