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THE HUMAN MICROBIOME

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The symbiotic relationship with the microbial flora inhabiting our bodies plays an immense role in maintaining our vitality. The microbiota protects us from pathogens, hardwires our immunity, and engages in the production of essential micronutrient components. The human microbiota encompasses several thousands of fungi, eubacteria, archaea and viruses, with eubacterial cells alone totaling over 10 trillion and outnumbering our body cells 100 to 1. Next generation sequencing has allowed researchers to comprehensively assess the diversity of microbial species in the human microbiota and to estimate their proportions with stunning accuracy. This has led to a breakthrough in our understanding of associations between human health and the microbiota. This review focuses on the current state of research on key microbial communities inhabiting the human body: those of the gastrointestinal and genitourinary systems. Less studied microbial communities colonizing the nose, nasopharynx, auditory canal, eye, and skin, as well as some others, are not included in the review.

Keywords: microbial community, gut flora, genitourinary, periodontal, metagenome, next generation sequencing

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МИКРОБИОМ ЧЕЛОВЕКА

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Симбиотическая микрофлора играет огромную роль в обеспечении здорового состояния нашего организма. Она защищает от патогенов, поддерживает иммунитет, обеспечивает производство важных компонентов питания. Микробиота человека включает, по всей видимости, несколько тысяч видов грибов, эубактерий, архей и вирусов. Суммарное количество клеток только эубактерий в составе микробиоты превышает десять триллионов, что в сто раз больше числа собственных клеток организма человека. С появлением методов высокопроизводительного секвенирования исследователи получили возможность очень точной и комплексной оценки всего микробного сообщества с глубиной до тысячных долей процента (по содержанию микроба). Это позволило выйти на новый уровень понимания взаимосвязи здоровья человека и состояния его микробиома. В данном обзоре представлено современное состояние исследований ключевых микробных биоценозов человека — пищеварительного и урогенитального трактов. Менее изученные биоценозы носа и носоглотки, слухового канала, глаз, кожи и ряд других в обзор не вошли.

Ключевые слова: микробный биоценоз, микрофлора кишечника, урогенитальный, пародонтальный, метагеном, высокопроизводительное секвенирование

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The gut microbiome

The human gut microbiota is one of the most actively studied microbial communities. It is incredibly complex in composition and interacts extensively with the human host. The role of the gut microbiota in pathology has been increasingly hypothesized, and the evidence to support those theories is still growing.

The human gut microbiota goes through a number of development stages until it is finally shaped. First, the fetus is colonized *in utero* by the bacteria coming from maternal sources, including the intestines, oral cavity and vagina [1]. Second, when going through the birth canal, the baby picks up another lot of its mother's microbes [1]. Besides, breast milk is not sterile and contains substantial amounts of bacteria,

such as *Streptococcus*, *Staphylococcus*, *Propionibacterium*, and *Bifidobacterium* [2]. The early postnatal gut microbiota is abundant with *Bifidobacterium* species [3] that thrive on human milk oligosaccharides. By the age of two, *Bifidobacterium* species become less abundant and the gut microbiota of the child starts to resemble that of an adult [3]. In infants born by caesarian section the composition of the gut microbiota in the first few months after birth is different from that of vaginally born children, which may be explained by the lack of contact with the vaginal microbiota of the mother, her exposure to antibiotics or the delayed onset of breastfeeding [3, 4].

The gut microbiota of adults encompasses over 600 microbial genera [5]. The *Firmicutes* and *Bacteroidetes* phyla together make up about 90 % of the microbial community, in which they are mainly represented by poorly culturable obligate anaerobes. In the European population *Firmicutes* are normally represented by *Faecalibacterium prausnitzii*, *Blautia*, *Dorea*, *Roseburia*, and *Coprococcus*. Intestinal *Bacteroidetes* are usually represented by *Bacteroides*, *Parabacteroides*, *Prevotella*, *Odoribacter*, *Barnesiella*, and *Alistipes* [5, 6]. A few percent of the gut microbiota of adult humans are made up of *Actinobacteria* and *Proteobacteria* [5, 7], the proportion of *Fusobacteria*, *Verrucombicrobia*, and methanogenic archaea *Euryarchaeota* is even smaller [5, 8].

Members of the gut microbiota have complex symbiotic and antagonistic relationship influencing the abundance of each group. Therefore, some of the compositional patterns - discrete and stable clusters called enterotypes – will occur more frequently in the population than their gradient forms. Researchers distinguish between three major enterotypes depending on whether the latter are rich in *Bacteriodes*, *Prevotella*, or *Ruminococcaceae*, respectively [5, 9]. Unfortunately, this classification cannot describe the real diversity of qualitative and quantitative characteristics of intestinal microbial communities. Gut microbiota composition is affected by dietary and smoking habits, age, body mass index, levels of hemoglobin and red blood cells in the blood, and exposure to antibiotics [5]. Diet has also been speculated to be a source of marked interpopulation differences in the microbiota composition [7, 8]. Although the term “dysbiosis” is readily used to describe the state of the microbial community that contributes to pathology, the “normality” of the gut microbiota composition is a matter of controversy.

The gut microbiome profusely synthesizes substances capable of entering the bloodstream and affecting distal organs and systems. Some researchers even called the gut microbiome a “virtual endocrine organ” [10, 11]. Members of the gut microbiota can secrete into the blood stream such agents as serotonin, gamma-aminobutyric acid, histamine, acetylcholine, dopamine, and noradrenaline [11]. They also produce a number of ligands that bind to the receptors of the innate and adaptive immune systems, such as flagellin, formyl methionine-containing peptides, lipopolysaccharides, and capsular polysaccharides (polysaccharide A of *Bacteroides fragilis*) [12].

There has been considerable focus on short-chain fatty acids, the end-products of carbohydrate fermentation carried out by microbial communities under anaerobic conditions of the intestines. These are usually acetic, propionic and butyric acids produced at a molar ratio of 3 : 1 : 1 [11, 13]. They are rapidly and efficiently absorbed in the intestines, with only 5 to 10 % excreted in feces [13].

Short-chain fatty acids exert their functions through a variety of mechanisms. First, they can be used by our body cells to harvest energy produced during oxidative phosphorylation. Butyric acid alone can cover 60 to 70 % of colonocytes’ energy needs [13]. Second, short-chain fatty acids inhibit histone deacetylase and thus down-regulate inflammation: they modulate transcriptional activity of NF-κB factors, reduce production of TNF-α and induce maturation of $FoxP3^+$ T_{reg}-cells [12]. Third, short-chain fatty acids can specifically bind to a few G-protein-coupled receptors, namely GPR41, GPR43 and GPR109A [12, 13]. These receptors are involved in regulating the growth and activities of microglia, dendritic cells and Tregs [12].

The range of short-chain fatty acids’ activities is not limited to their effect on the immune system. They also induce proliferation of intestinal goblet cells and stimulate mucin production [12]. Being a substrate for gluconeogenesis and lipogenesis, they participate in the regulation of carbohydrate and lipid metabolism in the liver [13]. They also have been shown to suppress appetite by stimulating secretion of leptin in adipocytes and inducing production of YY peptide and glucagon-like peptide-1 in the L-cells of the gastro-enteropancreatic endocrine system [13]. Indeed, one of the most important functions of the gut microbiome is to produce short-chain fatty acids essential for human health

The gut microbiota is also responsible for protecting the intestine from pathogen dissemination which can be controlled through competition for nutrients [14]. Another mechanism of colonization resistance is mediated by special antimicrobial proteins and peptides, the so-called bacteriocins, produced by the gut microbiota [15]. Antimicrobial activity is also conferred on secondary bile acids, products of dehydroxylation of primary bile acids by members of the gut microbial community, such as *Clostridium scindens* [16].

Among all human diseases *Clostridium difficile*-associated disease is most obviously linked to shifts in the gut microbiota composition. Its clinical signs may vary from mild diarrhea to lethal systemic inflammatory response syndrome [17]. *Clostridium difficile* is often present in the microbiota of healthy individuals; however, intake of broad-spectrum antibiotics that disrupt colonization resistance mechanisms prompts this species to proliferate uncontrollably producing toxins that glycosylate Rho GTPhase [16, 17]. There is evidence that oral intake of live probiotic cultures can be effective in preventing *Clostridium difficile*-associated disease in both children and adults [18]. One of the most effective treatments for this condition is fecal microbial transplantation. A microbial suspension prepared from the gut microbiota of a healthy donor is infused into the patient’s intestines by enema, colonoscopy, nasogastric or nasoduodenal tubes [19]. There are plans to create donor banks of the intestinal microbiota that could be used for autologous fecal transplantation should it be necessary (Fig. 1). Unlike probiotic-based therapies, this technique makes it possible to transplant the entire microbial community including its poorly cultured members.

One of the most dangerous diseases typically seen in premature infants is necrotizing enterocolitis, the acute inflammatory condition of the bowel complicated by necrosis of the intestinal wall, perforation and diffuse peritonitis. According to the most recent studies, necrotizing enterocolitis is associated with hyperresponsiveness of the innate immunity to microbial colonization of the bowel [21]. The inadequate immune response can be caused by the interaction between overexpressed Toll-like receptors 4 and lipopolysaccharides found in the cell wall of gram-negative bacteria [21]. Numerous studies have described compositional changes in the gut microbiota of infants observed prior to the onset of necrotizing enterocolitis: increased abundance of *Proteobacteria* and low

levels of *Firmicutes* and *Bacteroidetes* [22]. At the moment there are reasons to believe that some probiotics can reduce incidence of severe necrotizing enterocolitis and improve survival [21, 23].

A few residents of the gut microbiota are involved in the development of colorectal cancer and are frequently detected in tumor tissues [24]. For example, *Fusobacterium nucleatum* can promote tumor growth through direct or inflammation-mediated mechanisms. In particular, interaction between the FadA adhesin produced by this species and the surface protein E-cadherin triggers a cascade of β-catenin-dependent oncogenic and proinflammatory signaling pathways [24]. Some strains of *Escherichia coli* are also potentially oncogenic since they produce genotoxic pathogenic factors, such as low molecular weight colibactin and protein toxin CDT [24, 25].

There has been growing evidence that the gut microbiota is involved in the pathogenesis of many other diseases, such as type 1 diabetes [26], obesity [27] and autism [28], which encourages us to believe that our knowledge about the intestinal microbiome and its role in human health will continue to expand.

The microbiome of the oral cavity and periodontium

The oral cavity of humans teems with eubacteria, archaea, fungi and viruses — over 1000 different species in total. Residents of the oral microbiota have been linked to a wide range of conditions, including diseases of the oral cavity (caries and periodontal diseases), diabetes mellitus, cardio-vascular diseases, cancer, etc. It has been established that it is not the presence of a particular microbe that triggers disease progression but a combination of microorganisms inhabiting the oral cavity.

To study microbial communities of the oral cavity, the following types of samples are normally collected: saliva, soft deposits, sub- or supra gingival calculus, and periodontal pocket contents. In terms of composition, these communities, except that of the periodontal pocket, are highly unstable and largely depend on dental care intensity and type. For example, one of the studies in which next-generation sequencing techniques were used allowed the researchers to estimate relative abundance of microbial residents of the subgingival plaques: 1.0–13.5 % for *Actinobacteria*, 21.4–63.5 % for *Bacteroidetes*, 14.6–30.8 % for *Firmicutes*, 4.7–12.1 % for *Fusobacteria*, 2.6–22.9 % for *Proteobacteria*, 0.04–12.9 % for *Spirochaetes*, and 0.0004–0.84 % for *Synergistetes* [29].

One of the most stable ecological niches of the oral cavity is the periodontal pocket. It is isolated from the external environment and is hardly affected by regular dental care (Fig. 2).

There has been a lot of research indicating the connection between the composition of the periodontal pocket microbiota and caries or periodontitis [30, 31]. A few authors have demonstrated the connection between periodontal microbiota composition and conditions of the lower digestive tract [32, 33]. An association between the periodontal pocket microbiota and patient's sex has been established. For example, hypercolonization of periodontal tissues by *Porphyromonas gingivalis* correlates with the severity of chronic periodontitis in women, but not in men. In contrast, *Tannerella forsythensis* alone or together with *Treponema denticola* is the only periodontal pathogen whose predominance is statistically associated with chronic periodontitis in men [34].

In their work Zorina et al. [35] analyzed the abundance of bacterial species and genera in the periodontal microbiota of patients with aggressive periodontitis and healthy individuals. It was discovered that of all studied genera 6 were potentially capable of protecting the periodontium and 8 were potentially pathogenic and associated with the risk of aggressive (but not chronic) periodontitis. The researchers demonstrated significantly increased abundance of *Porphyromonas*, *Treponema*, *Synergistes*, *Tannerella*, *Filifactor*, *Ruminococcus*, *Parvimonas*, and *Mycoplasma*, of which three (*Porphyromonas*, *Treponema* and *Tannerella*) are conventionally considered periodontal pathogens. Interestingly, *Veillonella* was found

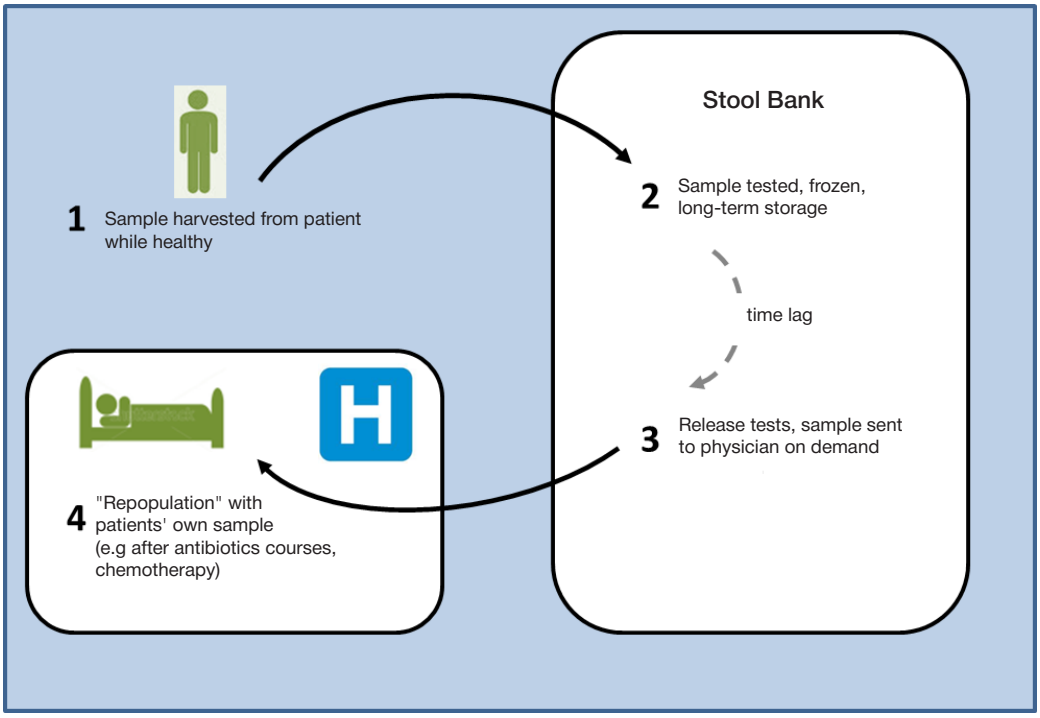

Fig. 1. Autologous stool banking for microbiota transplants (Olle, [20])

to dominate other microbiota residents in the control group, therefore it may be used as a criterion of periodontal health. The researchers also proposed to include *Streptococcus*, *Bergeyella*, *Granulicatella*, *Kingella* and *Corynebacterium* in the list of potential periodontal protectors [35].

The microbiome of the reproductive system

It has been long known that the microbiota of the female reproductive system is very diverse. Traditionally the focus was on the vaginal microbiota, but over the past few decades sufficient evidence was obtained to prove that other parts of the female reproductive tract, including the uterine cavity, are not sterile as well [36]. It is becoming clear that the microbiota extends up and over the endometrial cavity. According to some researchers, bacteria can also be found in the fallopian tubes of healthy women.

Studies of associations between the microbiota of the reproductive tract and successful fertilization/normal pregnancy are starting only now. So far, the association has been established between clinically manifested infection, inflammation and defective reproductive function. Inflammation triggers secretion of proinflammatory cytokines and growth factors produced by immune cells that are activated in response to pathogen invasion. Even small changes in the microbiome can entail changes in the surrounding tissue that are normally less evident but can be clinically significant [37].

The normal vaginal microbiota is dominated by lactobacilli [38] that have probiotic properties and inhibit growth of other bacteria. *Lactobacilli* produce large amounts of H_2O_2 and are believed to be highly beneficial. This leads us to understand that direct interaction between microbes and the surrounding tissues is possible but does not have to be the rule, and that perhaps the primary function of certain microbiome components is to inhibit expansion of other microbiota residents.

The microbiota of the reproductive system is not a mere aggregation of free-floating bacteria. In many cases these bacteria produce complex 3D biofilm structures, sometimes multilayered, consisting of polysaccharides, nucleic acids and proteins, serving as a protective coat. Sometimes these biofilms prevent the immune system from detecting a pathogen and diminish positive effects of antimicrobial treatment [39].

Biofilms usually occur in the vagina but can extend into the endometrial cavity [39] or even further upwards into the fallopian tubes. Although no definite conclusions have been made so far about the role of such biofilms in the pathology of the reproductive system, one should have a clear understanding that the connection between the microbiome and the reproductive system may not be determined solely by the abundance or the lack of certain bacterial species.

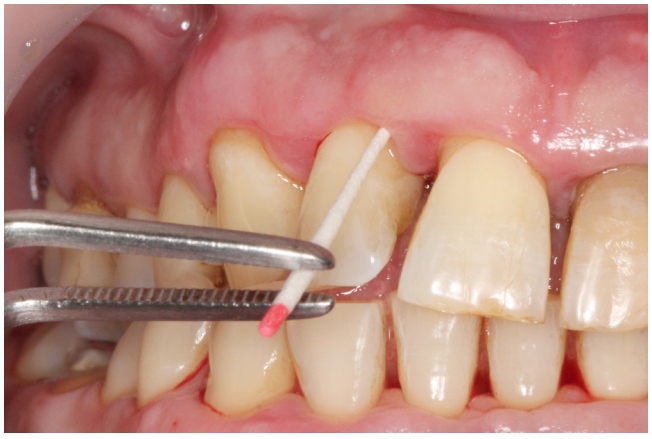

Fig. 2. Collecting a periodontal pocket sample [Photo courtesy of Nelly Aimadinova of Central Research Institute of Dental and Maxillofacial Surgery, Moscow, Russia]

The microbiome can affect gametogenesis. It was found that some bacteria can undermine follicular development and suppress follicular response to gonadotropin [36]. Similarly, some bacteria produce a negative effect on the reproductive system of men. Even slight changes in the microbiome can impact semen quality. The microbiome of the male reproductive system turns out to be more complex than it was thought before. As our knowledge about the microbiomes of female and male reproductive systems is expanding, we are discovering new therapeutic targets.

The vaginal microbiome

Studies of healthy vaginal microbiota were carried out under the Human Microbiome Project [38]. Samples of 113 healthy female volunteers were used to characterize three microbial communities of the vagina: those inhabiting the vaginal introitus, the midpoint, and the posterior fornix. The samples were analyzed using 16S rRNA pyrosequencing. Alpha and beta diversities (i. e., in one individual and between different individuals, respectively) of vaginal microbial communities were described. Interestingly, the study yielded unexpected results. It was established that in comparison with other body parts, such as mouth or skin, the reproductive system harbors a microbiota with the lowest alpha and a very low beta diversities in terms of bacterial phyla [38]. Besides, the samples obtained from different regions of the vagina did not vary much in bacteria species and were dominated by *Lactobacillus*. Samples of the same donor collected at different time points varied less than samples of different individuals, indicating that the vaginal microbiota is stable over time. Vaginal microbial communities of healthy women are relatively simple in composition compared to communities inhabiting other body parts, which means that health and pathology may be associated with certain shifts in the microbiota [39].

The Human Microbiome project recruited healthy women to explore “healthy” microbiomes. There were other projects in which the association between the vaginal microbiota and infertility in women was studied. In one of such studies, a bacterial culture method was applied to prospectively analyze 152 patients who had undergone In Vitro Fertilization (IVF) treatment [40]. Samples of 133 (87.5 %) women were positive for one or more microorganisms; 19 (12.5 %) samples were negative for any microbial contamination. The most common bacteria detected in the samples were *Lactobacillus spp.*, *Staphylococcus spp*. and *Enterobacteriaceae*, including *E. coli*, *Klebsiella* and *Proteus*. Successful fertilization was observed in 12.4 % of patients positive for one or more bacterial species and in 14 % of women tested negative for any bacteria (p <0. 001). Besides, patients who tested positive for *Enterobacteriaceae* and *Staphylococcus* were found to have lower pregnancy rates than those tested negative. Though this study provides some insight into the microbiota composition during IVF treatment, it also points out limitations of culture methods in microbiota assessment. The fact that 12.5 % of patients tested absolutely negative for bacterial contamination indicates that methods based on culture isolation seriously underestimate the abundance and diversity of microbiota residents during IVF.

Using 16S RNA sequencing, the researchers described the vaginal microbiota of the infertile patient who had undergone

IVF treatment [40]. Poor bacterial diversity was shown to be associated with higher probability of live birth. To date, there are effective molecular and biological techniques that facilitate studies of the vaginal microbiome [41]. Robust data have been obtained on the quality of vaginal microbiota in pathology [42, 43], but research of the microbiome still goes on.

The microbiome of the uterus

Until recently it was believed that microbial colonization of the upper genital tract occurring by the ascending pathway from the vagina through the cervix could be related only to a pathological condition. Cervical mucus contains high levels of proinflammatory cytokines, immunoglobulins and antimicrobial peptides and acts as a protective barrier, which is why the uterine cavity of healthy women was long considered sterile [44–48]. However, upward transport is quite possible in a healthy reproductive tract. For example, 2 minutes after 1–2 ml radiolabeled sperm-sized macroaggregates of human serum albumin were placed into the posterior vaginal fornix, they were observed in the uterus [49].

Early studies of the uterine microbiota were carried out using culture methods, which have certain limitations described above (See *The vaginal microbiome*). In a recent study, samples of 58 women undergoing hysterectomy were tested for 12 bacterial species using quantitative PCR assays [50]. Vaginal swabs were collected before hysterectomy, while uterine swabs were collected after the surgery. Colonization of the upper genital tract by at least one bacterial species was confirmed in 95 % of cases. The most frequently observed species were *Lactobacillus* and *Prevotella*. Of note, the average number of bacteria in the upper genital tract was lower than in the vagina, by 2–4 $\log_{10}$ RNA gene copies per swab. This means that either the cervix acts as a filter for ascending microorganisms or the immune system suppresses their upward transport; a combination of both mechanisms is also possible.

The microbiome of ovarian follicles

Human follicular fluids are readily culturable and are inhabited by microbes in many patients, as shown by a number of studies. In those studies, some of the samples were collected from the follicular aspirate during transvaginal oocyte retrieval, others were obtained through laparoscopy [51–54]. It is unclear whether the bacteria cultured from the collected samples were in the follicles prior to oocyte retrieval or the follicular fluid was contaminated during follicular aspiration [52, 54]. Some authors believe that microbes can be classified as colonizing or contaminating based on the comparison of microbiota composition of the sample with the bacteria detected on the surface of the puncture needle [54, 55]; if the follicle contains unique species, they should be considered colonizing. This approach to classification, however, does not account for the cases when a potential pathogen moves upwards from the uterus to the upper genital tract colonizing this region. The follicular fluid was found to contain microbes typical for the healthy microbiota of the vagina (*Lactobacillus spp.*), gastrointestinal tract (*Bifidobacterium spp.*, *Enterobacteriaceae*, *Streptococcus agalactiae*), skin (*Staphylococcus spp.*) and oral mucosa (*Streptococcus spp.*), which supports the hypothesis that the follicular fluid does not always get contaminated during oocyte retrieval and can be colonized before this procedure [56]. So far, there has been no research to assess the vaginal, cervical, endometrial, fallopian, follicular and peritoneal microbiomes in parallel.

The microbiome of the reproductive system of men

Because of small sample sizes, there are only scanty data on the composition of the healthy urethral microbiota of men. For example, in a group of 33 men without urethritis, only the presence of *Staphylococcus epidermidis*, *Corynebacterium spp.*, *Lactobacilli*, *Haemophilus vaginalis*, and *alpha-hemolytic streptococci* was detected by a standard culture method [57]. In another study 16S rRNA gene sequencing was used to analyze 9 samples of first pass urine of men who had no clinical signs of urethritis or sexually transmitted infections; the most frequently detected bacteria were Corynebacterium, Lactobacilli and Streptococci [58]. In the coronal sulcus of uncircumcised men, whose sexual partners had no bacterial vaginosis, non-culture methods revealed the presence of *Corynebacterium*, *Lactobacillus* and *Staphylococcus* [59]. Thus, unlike women of reproductive age whose microbiota is dominated by *Lactobacillus* [60], men's microbiota of the urethra is not dominated by any particular species, and bacterial communities are usually complex [58].

Traditionally, research of semen microbiota was carried out using the culture method. It was successfully applied to discover associations between acute or chronic prostatitis and some infections, including gonorrhea and chlamydia. But recently metagenomics was introduced to describe semen microbiomes and conduct the traditional semen analysis. Hou et al. analyzed 77 samples collected from 58 infertile men and 19 healthy sperm donors [61]. Patients were divided into 6 groups based on the similarities of microbiota composition and diversity of taxa. It was shown, however, that their semen had very similar characteristics. Further analysis showed that only *Anaerococcus* was significantly associated with compromised sperm quality. Recently Weng et al. have conducted a similar research using 96 samples [62], of which 60 had one or more defects in semen parameters. The rest 36 samples were normal and used as control. *Pseudomonas*, *Lactobacillus* and *Prevotella* were prevailing microorganisms. The most interesting association was established among these taxa and the quality of corresponding sperm samples. In the samples dominated by *Lactobacillus* the proportion of healthy sperm cells was very high. It indicates that some *Lactobacillus* species inhabiting the male reproductive tract can exert probiotic activities protecting the host from pathogens, as is the case with the female reproductive system.

The conducted studies raise questions rather than answer them. They demonstrate the associations between the clinical signs of the pathology and microbiota composition. It is unknown what does harm to sperm cells: shifts in the microbiome that shape the environment or differences in semen properties that create favorable conditions for various bacteria. However, these first results are very important and urge us to collect more data. Some authors indicate that similar studies are being carried out at the moment.

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IDENTIFICATION OF AMINOGLYCOSIDE PHOSPHOTRANSFERASES OF CLINICAL BACTERIAL ISOLATES IN THE MICROBIOTA OF RUSSIANS

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Antibiotic resistance is one of the biggest threats to modern medicine. Response to antimicrobial treatment is seriously disrupted by aminoglycoside phosphotransferases (Aph) — enzymes produced by bacteria. The *aph* genes were annotated in many bacterial species, including commensals of the gut microbiota that can transfer these genes to clinically important strains. For this study we prepared a catalog of 21 *aph* genes. The *in silico* analysis of 11 intestinal microbiomes of healthy Russians revealed the presence of 3 cataloged *aph* genes in 7 microbiota samples, namely *aph(3'')-Ib*, *aph(3')-IIIa* and *aph(2'')-Ia*. The most frequent was the *aph(3')-IIIa* gene detected in 6 metagenomes. Of note, this gene was first discovered in Enterococcus faecalis, but in this study we observed it in sequences typical for commensal *Ruminococcus obeum* and opportunistic *Enterococcus faecium*, *Roseburia hominis*, *Streptococcus pyogenes* and *Staphylococcus epidermidis*. Similarly, *aph(2'')-Ia* originally present in *E. faecalis* was detected in a sequence typical for *Clostridium difficile*. Our findings are consistent with the reports on the strong association between the geographical origin of the individual and frequency of *aph* genes. We suggest that clinical examination should include antibiotic sensitivity tests run not only on the causative agent, but also on the gut microbiota, for a better treatment outcome.

Keywords: antibiotic resistance, aminoglycoside phosphotransferase (Aph), clinical isolates of bacteria, human gut microbiota

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ИДЕНТИФИКАЦИЯ АМИНОГЛИКОЗИДФОСФОТРАНСФЕРАЗ КЛИНИЧЕСКИХ ШТАММОВ БАКТЕРИЙ В МИКРОБИОТЕ ЖИТЕЛЕЙ РОССИИ

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Устойчивость бактерий к антибиотикам является одной из самых серьезных проблем в современной медицине. Эффективность антимикробной терапии снижается вследствие работы бактериальных ферментов — аминогликозидфосфотрансфераз (Aph). Гены *aph* аннотированы в геномах многих бактерий, в том числе комменсалов микробиоты кишечника, из геномов которых они могут попадать в геномы клинически значимых штаммов. Анализ *in silico* 11 метагеномов кишечника здоровых людей из России показал наличие в 7 образцах микробиоты 3 генов *aph* из 21, включенного в каталог, составленный для исследования: *aph(3'')-Ib*, *aph(3')-IIIa* и *aph(2'')-Ia*. Наиболее распространенным оказался ген *aph(3')-IIIa*, найденный в 6 исследованных метагеномах. Важно, что этот ген впервые был обнаружен у *Enterococcus faecalis*, но в данной работе он был идентифицирован в генетическом окружении, характерном для комменсальной бактерии *Ruminococcus obeum* и условно-патогенных бактерий *Enterococcus faecium*, *Roseburia hominis*, *Streptococcus pyogenes* и *Staphylococcus epidermidis*. То же наблюдали для гена *aph(2'')-Ia*: он был обнаружен для *Clostridium difficile*, а не для *E. faecalis*. Полученные результаты согласуются с литературными данными, указывающими на значимое влияние географического происхождения людей на распространенность *aph*-генов. Также, учитывая данные исследования, представляется обоснованным при клиническом обследовании пациентов с инфекционными заболеваниями и назначении антибиотиков для их лечения анализировать антибиотикорезистентность не только бактерии-возбудителя, но и микробиоты пациента.

Ключевые слова: устойчивость к антибиотикам, антибиотикорезистентность, аминогликозидфосфотрансферазы, Aph, клинические штаммы бактерий, микробиом, микробиота, кишечник человека

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At least 2 million people in the USA become infected with antibiotic-resistant bacteria every year, and at least 23,000 people die of these bacterial infections [1]. The growing antibiotic resistance of human pathogens is a serious threat to global health and has a significant impact on the environment. According to Antibiotic Resistance Genes Database (ARDB) [2], 13,293 antibiotic resistance genes of microorganisms have been discovered so far. Transfer of genetic elements between bacteria through intricate routes in mixed microbial communities promotes dissemination of resistance genes [3].

The human gut is home to about 10^{14} microbial cells and approximately 1000 microorganisms [4]. It is a dynamic reservoir of antibiotic resistance genes termed the resistome [5]. Antibacterial treatment has a significant impact on the gut resistome: it stimulates horizontal gene transfer and exerts selective pressure on its members [6]. Studies of gut microbiota residents resistant to antibiotics show that commensals of the human gut can also be a source of resistance genes for other bacteria, including pathogenic strains [7].

Studies of antibiotic resistance employ various cutting-edge technologies and methods, such as next generation sequencing, bioinformatic analysis, or analytical chemistry, making it possible to identify up to 30 gene clusters associated with antibiotic resistance [8]. Researchers of the Center for Genome Sciences and Systems Biology, Washington University School of Medicine, analyzed genes responsible for resistance to 18 clinically relevant antibiotics across ecologies. The bioinformatic analysis identified genes conferring resistance to two antibiotics widely used in the clinical setting and agriculture: β-lactams and tetracyclines [9].

Antimicrobial therapies can be seriously disrupted by aminoglycoside phosphotransferases (Aph) [10]. Genes that encode these enzymes were first discovered in plasmids and mobile elements of clinical strains of gram-positive and gram-negative bacteria [11]. As demonstrated by the phylogenetic analysis of Aph of clinical strains and strains producing aminoglycoside antibiotics [12], aminoglycoside phosphotransferases can be organized in 7 groups depending on the enzyme-modified position of the hydroxyl group of the antibiotic: Aph(2''), Aph(3'), Aph(3''), Aph(4), Aph(6), Aph(7'') and Aph(9).

Aph-encoding genes have been annotated in many bacterial genomes, including non-pathogenic strains of the gut microbiota from where they can transfer to clinical strains [13]. Metagenomic DNA isolated from the human neonatal gut was shown to carry multiple genes conferring resistance to aminoglycosides and β-lactams [14].

A comparative study of 832 human gut metagenomes obtained from the residents of 10 different countries (England, Finland, France, Italy, Norway, Scotland, USA, Japan, China, and Malawi) established that the diversity of resistance genes was largely dependent on the geographical origin of the participant [15].

The spread of aph genes was studied in many laboratories worldwide. The *aac(6')-Ie-aph(2'')-Ia* gene was found to be the most prevalent gene of enterococcal aminoglycoside resistance; it was detected in 26 out of 27 isolates obtained from patients of an Iranian hospital [16]. The epidemiologic study of 543 clinical strains isolated from Japanese patients showed that of 12 studied genes of aminoglycoside-modifying enzymes, one — the *aph(2'')-Ie* gene- was isolated from 3 strains of *Enterococcus faecium* and another one –*ant(9)-Ia* — was detected in *E. faecalis*, *E. faecium* and *E. avium*. Nucleotide sequences of *ant(9)-Ia* in these 3 enterococci were identical to those of *Staphylococcus aureus* and were harbored on transposon Tn554 [17]. Because aminoglycosides are often used to treat staphylococcal infections, a study was carried out to estimate the prevalence of aminoglycoside resistance among methicillin-resistant strains of *S. aureus* isolated from patients of an Iranian hospital. Genes *aac(6')-Ie-aph(2'')*, *aph(3')-IIIa* and *ant(4')-Ia* were detected in 134 (77.0 %), 119 (68.4 %) and 122 (70.1 %) isolates, respectively [18].

In light of the above, identification of aminoglycoside phosphotransferases in the gastrointestinal metagenomes of Russian residents becomes a pressing issue.

METHODS

Sample preparation and DNA sequencing

We studied the gut microbiota of 11 healthy individuals of different sex and age, all residents of Moscow and Tver, Russia. Stool samples were collected using standard techniques [19]. Samples were frozen at –80 °C until further analysis.

DNA was extracted from weighted amounts of frozen stools using the QIAamp Fast DNA Stool Mini kit (Qiagen, Germany) according to the vendor's protocol with optimized lysis conditions for microbial DNA extraction (Isolation of DNA from Stool for Pathogen Detection, Qiagen, USA). The concentration of the obtained DNA was measured using the Qubit Fluorometer (Invitrogen, USA). The obtained genomic DNA was fragmented using the Covaris M220 focused ultrasonicator (Covaris, USA) to achieve fragment length between 100 and 700 b. p. (average size was ~350 b. p.).

Libraries for further sequencing were prepared using the NEBNext Ultra DNA Library Prep Kit for Illumina (NEB, UK). Fragments ranging from 250 to 500 b. p. (adapter sequences included) were selected for further sequencing. Quality control of the obtained libraries was performed on the Agilent TapeStation (Agilent Technologies, Germany); the libraries were mixed in equimolar amounts. Adapter sequences used at library prep step were as follows: Read1 (AGATCGGAAGAGCACACGTCTGAA CTCCAGTCACNNNNNNATCT CGTATGCCGTCTTCTGCTTG) and Read2 (AGATCGGAAGAGCGTCGTGTAGGGAAAGAG TGTAGATCTCG GTGGTCGCCGTATCAT), where NNNNNN is a 6-nucleotide index unique for each sample. After quality control was performed and library molecules were counted by quantitative PCR, the libraries were sequenced on one lane of Illumina HiSeq 4000 (101 cycles per each fragment's end) using the HiSeq 4000 SBS sequencing kit ver. 1 (Illumina, USA). FASTQ files were obtained using bcl2fastq v2.17.1.14 Conversion Software (Illumina). Quality scores were encoded as Phred 33. The obtained metagenomes were uploaded to the Sequence Read Archive (SRA) NCBI. They are presented in Table 1.

Quality control of metagenomic libraries and read assembly

Quality control of the resulting metagenomic libraries was performed using FastQC [20]. Read trimming was done using trimmomatic [21]. Contaminating host DNA was filtered by aligning the metagenomic reads against the human genome. Alignment was performed using Bowtie2 [22]. The metagenomic reads were assembled into contigs using SPAdes [23]. Description of the assembled reads is provided in Table 2.

Compiling a catalog of aminoglycoside phosphotransferases-encoding genes

Drawing upon the literature [12], we compiled a catalog of aminoglycoside phosphotransferase-encoding genes

isolated from the clinical strains of *Acinetobacter baumannii*, *Alcaligenes faecalis*, *Bacillus circulans*, *Burkholderia pseudomallei*, *Campylobacter jejuni*, *Enterococcus faecalis*, *Escherichia coli*, *Enterococcus casseliflavus*, *Enterococcus faecium*, *Legionella pneumophila*, and *Pseudomonas aeruginosa*. The catalog listed 21 gene. We also compiled a catalog of amino acid residues encoded by the selected Aph genes.

Metagenomic analysis

A Perl script was written to run the BLASTX search for aminoglycoside phosphotransferase genes in the assembled contigs and to filter the results by 2 parameters: homology and relative alignment length. The search was performed in the catalog of 31 amino acid sequences prepared in advance. Sequence alignments generated by BLASTIX were filtered by homology and relative alignment length. Relative alignment length was calculated as

$$L_{relative} = \frac{L_{alignment}}{L_{sequence}},$$

where $L_{alignment}$ is the length of the obtained alignment and $L_{sequence}$ is the length of the reference amino acid sequence from the catalog. We did not intend to screen the samples for new aminoglycoside phosphotransferase genes, therefore for homology the minimal value was set to 90 %, and the minimal alignment length was set to 80 %. To profile the species present in the studied samples, MetaPhlAn2 was used [24].

RESULTS

Compiling a catalog of aminoglycoside phosphotransferase genes of clinically relevant strains

Depending on the position of the enzyme-modified hydroxyl group of the antibiotic, aminoglycoside phosphotransferases were distributed into 7 subgroups: Aph(2''), Aph(3'), Aph(3''), Aph(4), Aph(6), Aph(7''), and Aph(9). The catalog of genes of clinical strains was prepared by summing up the data from the review [12]. The catalog of aminoglycoside phosphotransferase-encoding genes of clinically relevant bacterial strains is provided in Table 3.

Screening Russian metagenomes for aminoglycoside phosphotransferase genes

Using the Perl script, we analyzed gut metagenomes of 11 healthy Russian individuals. The results are presented in Table 4. It total, we identified 3 *aph* genes in 7 metagenomes. All genes were identified with 100 % homology. Of these 3 genes, the most prevalent was gene aph(3')-IIIa: it was missing in only one metagenome (D5F). Two *aph* genes, namely *aph(2'')-Ila* and *aph(3')-IIIa*, were present only in metagenome D12F. Gene *aph(3'')-Ib* was detected in only one metagenome (D5F).

The studied metagenomes were profiled for species diversity using MetaPhlAn2. Reads unambiguously assigned to bacterial species were aligned against metagenomic contigs using Bowtie2. Thus, contigs that carried aminoglycoside

Table 1. The studied metagenomes

№	Sample	Sex	Age, years	Region	Genbank ID
1	4B_S2	F	34	Tver, Russia	SRX1870055
2	12_S1	F	28	Tver, Russia	SRX1878777
3	D3F	M	15	Moscow, Russia	SRX2672491
4	D4F	M	15	Moscow, Russia	SRX2672492
5	D5F	M	15	Moscow, Russia	SRX2672493
6	D6F	M	15	Moscow, Russia	SRX2672494
7	D11F	M	15	Moscow, Russia	SRX2672495
8	D12F	F	15	Moscow, Russia	SRX2672496
9	D13F	F	15	Moscow, Russia	SRX2672497
10	DG_S1	F	28	Tver, Russia	SRX1869842
11	HG550	F	6	Tver, Russia	SRX1869839

Table 2. Description of the assembled reads

№	Sample	Contig length, MBases	Maximal contig length, b. p.	N50, b. p.
1	4B_S2	73	50917	2790
2	12_S1	160	111721	3800
3	D3F	106	855598	9284
4	D4F	237	433763	5677
5	D5F	140	517131	21016
6	D6F	238	544506	5742
7	D11F	46	1671967	7207
8	D12F	147	545374	7999
9	D13F	317	643760	12617
10	DG_S1	208	125246	2621
11	HG550	82	69816	3121

phosphotransferase genes [Kovtun AS, unpublished] could be assigned to certain species. Results of the bioinformatic analysis are presented in Table 5.

DISCUSSION

The *in silico* analysis of 11 gut metagenomes of healthy Russians revealed the presence of aminoglycoside phosphotransferases in 7 metagenomes. Of 21 aph genes previously isolated from the clinical strains of *Acinetobacter baumannii*, *Alcaligenes faecalis*, *Bacillus circulans*, *Burkholderia pseudomallei*, *Campylobacter jejuni*, *Enterococcus faecalis*, *Escherichia coli*, *Enterococcus casseliflavus*, *Enterococcus faecium*, *Legionella pneumophila*, and *Pseudomonas aeruginosa* listed in our aph catalog (Table 3), only 3 were found in the studied samples. Those are: *aph(3'')-Ib*, *aph(3')-IIIa* and *aph(2'')-Ia*. The most frequently occurring gene was *aph(3')-IIIa* (CAA24789) detected in 6 samples. This gene was previously discovered in *E. faecalis* and confers resistance to kanamycin. Gene aph(3'')-Ib (AAA26442) previously isolated from *E. coli* and associated with streptomycin resistance and gene aph(2'')-Ia (AAA26865) previously isolated from *E. faecalis* and associated with tobramycin resistance were observed in only one studied metagenome (Table 3).

Interestingly, the analysis of contigs that harbor aminoglycoside phosphotransferase-encoding genes revealed

Table 3. The catalog of aminoglycoside phosphotransferase-encoding genes of clinically relevant bacterial strains

Gene name	GenBank entry	Bacteria*	Gene location	Aminoglycoside resistance
aac(6')-Ie-aph(2")-Ia	AAA26865	*Enterococcus faecalis*	Chromosome	Tobramycin
aph(2'')-IIa	AAK63040	*Escherichia coli*	Chromosome	Kanamycin, gentamicin
aph(2'')-IIIa	AAB49832	*Enterococcus gallinarum*	Chromosome	Gentamicin
aph(2'')-IVa	AAC14693	*Enterococcus casseliflavus*	Chromosome	Gentamicin
aph(2'')-Ie	AAW59417	*Enterococcus faecium*	Chromosome	Gentamicin
aph(3')-Ia	CAA23656	*Escherichia coli*	Transposon Tn903	Kanamycin
aph(3')-Ib	AIL00451	*Pseudomonas aeruginosa*	Chromosome	
aph(3')-IIa	CAA23892	*Escherichia coli*	Transposon Tn5	Neomycin
aph(3')-IIb	AAG07506	*Pseudomonas aeruginosa*	Chromosome	Kanamycin, neomycin, butirosin, seldomycin
aph(3')-IIIa	CAA24789	*Enterococcus faecalis*	Chromosome	Kanamycin
aph(3')-IVa	P00553	*Bacillus circulans*	Transposons Tn5 and Tn903	Kanamycin, neomycin
aph(3')-VIa	CAA30578	*Acinetobacter baumannii*	Chromosome	Kanamycin, amikacin
aph(3')-VIb	CAF29483	*Alcaligenes faecalis*	Transposon Tn5393	Kanamycin, streptomycin, amikacin
aph(3')-VIIa	P14508	*Campylobacter jejuni*	Chromosome	Kanamycin, neomycin
aph(3')-VIIIa	P14509	*Escherichia coli*	Plasmid RP4	Kanamycin, neomycin
aph(3'')-Ib	AAA26442	*Escherichia coli*	Plasmid RSF1010	Streptomycin
aph(4)-Ia	P00557	*Escherichia coli*	Plasmid pJR225	Hygromycin
aph(4)-Ib	CAA52372	*Burkholderia pseudomallei*	Chromosome	Hygromycin
aph(6)-Ic	CAA25854	*Escherichia coli*	Transposon Tn5	Streptomycin
aph(6)-Id	AAA26443	*Escherichia coli*	Plasmid RSF1010	Streptomycin
aph(9)-Ia	AAB58447	*Legionella pneumophila*	Chromosome	Spectinomycin

Note. * — a microorganism the gene was first isolated from.

Table 4. Aminoglycoside phosphotransferase genes identified in the studied metagenomes

Gene name	Metagenome ID										
	4B_S2	12_S1	D3F	D4F	D5F	D6F	D11F	D12F	D13F	DG_S1	HG550
aph(2'')-Ia	–	–	–	–	–	–	–	+	–	–	–
aph(3'')-Ib	–	–	–	–	+	–	–	–	–	–	–
aph(3')-IIIa	–	–	+	+	-	+	–	+	+	–	+

Table 5. Diversity of species in the studied metagenomes with identified aminoglycoside phosphotransferase genes

Metagenome	Contig length, b.p.	*aph(2'')-Ia*	*aph(3'')-Ib*	*aph(3')-IIIa*
D3F	3389	–	–	*Enterococcus faecium*
D4F	6439	–	–	*Ruminococcus obeum*
D5F	1422	–	*Escherichia coli*	–
D6F	979	–	–	*Enterococcus faecium*
D12F	5607 (для гена *aph(3')-IIIa*); 4407 (для гена *aph(2'')-Ia*)	*Clostridium difficile*	–	*Roseburia hominis*
D13F	4356	–	–	*Streptococcus pyogenes*
HG550	2242	–	–	*Staphylococcus epidermidis*

the presence of the latter in the genomes of other bacterial species. For example, the *aph(3')-IIIa* gene was detected in a sequence typical for commensal *Ruminococcus obeum* and opportunistic *E. faecium*, *Roseburia hominis*, *Streptococcus pyogenes* and *Staphylococcus epidermidis*, but not for *E. faecalis*. Gene *aph(2'')-Ia* was detected in *Clostridium difficile*, but not in *E. faecalis* (Tables 3, 5). Although this gene was the most prevalent in enterococci in the study [16], we observed it in only one studied sample in the non-enterococcal sequence. Genes *aph(2")-Ia* and *aph(3')-IIIa* were previously reported in methicillin-resistant strains of *Staphylococcus aureus* [17]. However, in the studied Russian metagenomes aph(3')-IIIa was present in the sequence typical for *Staphylococcus epidermidis*, while *aph(3'')-Ib* was detected in *E. coli*.

These results are consistent with the results of comparative analyses conducted worldwide: age, sex and health do not have any significant impact on the antibiotic resistance of the gut microbiota, while the geographic origin does [15]. Rare occurrence and poor diversity of *aph* genes in Russian metagenomes may indicate that gut microbiota composition is specific to a particular region and that individuals whose microbiomes were analyzed in our study rarely resort to aminoglycoside therapies. On the other hand, missing *aph* genes in anaerobic bacteria that dominate the gut microbiota may be explained by the absence of cytochrome-mediated transport [25]. It is also important that the microbiome of a healthy individual harbors opportunistic bacteria carrying *aph* genes.

CONCLUSIONS

Previously isolated from clinical bacterial strains, genes *aph(3'')-Ib*, *aph(3')-IIIa* and *aph(2'')-Ia* were found in 7 microbiota samples of 11 healthy Russians. Gene aph(3')-IIIa prevailed. The genes detected in the samples are carried by opportunistic bacteria: *Enterococcus faecium*, *Roseburia hominis*, *Clostridium difficile*, *Escherichia coli*, *Streptococcus pyogenes*, and *Staphylococcus epidermidis*. Two of them — *E. coli* and *E. faecium* — belong to a group of 12 highly dangerous bacteria, according to the World Health Organization. Therefore, we believe it reasonable to run antibiotic resistance tests on both the causative agent and patient's microbiota before deciding on the antibiotic treatment for patients with bacterial infections.

This work is the first to study the spread of antibiotic resistance genes of the gut microbiota of Russians. Further PCR-based search should be conducted to identify other clinically relevant resistance genes.

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SERINE/THREONINE PROTEIN KINASES OF BACTERIA ARE POTENTIAL TARGETS FOR REGULATION OF HUMAN MICROBIOTA COMPOSITION

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Serine/threonine protein kinases (STPKs) of bacteria are involved in signal transduction, cell growth and division, biofilm formation and virulence regulation. They are found in both pathogenic microbes and symbiotic residents of the human microbiota. Previously we proposed a classification scheme for STPKs of gram-positive bacteria based on the signature sequence of 9 amino acid residues in the ATP-binding pocket. Accordingly, protein kinases and bacterial species that contained those kinases were divided into 20 groups. We hypothesized that STPKs with identical signatures would interact with the same low-molecular-weight compounds that could be used as selective inhibitors of STPK to suppress growth and virulence of certain residents of the human gut microbiota (GM). GM represented by over 400 bacterial species is critical in maintaining homeostasis in the human body. In healthy individuals GT composition is balanced in terms of genera/species abundance. Shifts in the GT composition are thought to trigger pathology. In this connection various approaches are being developed to regulating the composition of the human microbiota. This article proposes the use of bacterial STPK inhibitors as “gentle” therapeutic agents for correcting taxonomic imbalances of GM triggered by non-infectious diseases and reducing virulence of pathogenic microbes with minimal impact on human protein kinases.

Keywords: gut microbiota, serine-threonine protein kinases, classification, selective inhibitors, gram-positive bacteria

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СЕРИН-ТРЕОНИНОВЫЕ ПРОТЕИНКИНАЗЫ БАКТЕРИЙ — ПОТЕНЦИАЛЬНАЯ МИШЕНЬ ДЛЯ РЕГУЛЯЦИИ СОСТАВА МИКРОБИОТЫ ЧЕЛОВЕКА

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Серин-треониновые протеинкиназы (СТПК) участвуют в передаче сигналов у бактерий, вовлечены в процессы роста и деления клетки, образование биопленок и формирование вирулентности. Они встречаются как у патогенных бактерий, так и у симбионтов микробиоты человека. Нами была разработана классификация СТПК грамположительных бактерий, в основе которой лежит сигнатура из 9 аминокислотных остатков, расположенных в области связывания аденина. На основе сигнатуры протеинкиназы и содержащие их роды и виды бактерий были разделены на 20 групп. Было выдвинуто предположение, что СТПК с одинаковой сигнатурой будут взаимодействовать со сходными низкомолекулярными веществами, которые могут быть использованы в качестве селективных ингибиторов СТПК для снижения скорости роста и вирулентности определенных групп бактерий кишечной микробиоты (КМ) человека. КМ, представленная более чем 400 видами бактерий, играет ключевую роль в поддержании гомеостаза организма человека. В норме состав КМ сбалансирован по видам и родам, но при различных заболеваниях таксономический баланс нарушается. Предполагается, что такого рода изменения могут являться триггерами заболеваний. В связи с этим разрабатываются различные подходы по регуляции состава микробиоты человека. В статье предложена концепция, основанная на использовании ингибиторов бактериальных СТПК в качестве «мягкой силы» для коррекции таксономического дисбаланса КМ, вызванного неинфекционными заболеваниями, а также для воздействия на патогенные микроорганизмы (снижения их вирулентности) при минимальном воздействии на протеинкиназы человека.

Ключевые слова: кишечная микробиота, серин-треониновые протеинкиназы, классификация, селективные ингибиторы, грамположительные бактерии

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Interactions between the gut microbiota and the host

The human microbiota is a microbial community that inhabits various body surfaces, such as the skin, the genitourinary system or the gastrointestinal tract (GIT), etc. Shaped by evolution, it has become an essential body organ. Our relationships with the microbiota are symbiotic. About 60 % of our symbionts live in the large intestine constituting the largest

microbial population of the body [1, 2]. The functions and the composition of the microbiota depend on age, sex, diet, environmental factors and social conditions, individual's health and the use of medications, including antibiotics [3, 4].

The microbiota in general and the gut microbiota in particular can significantly affect human health. Being the largest microbial reservoir of the body, the microbiota of the small and large intestines can influence the function of the GIT and other vital organs and systems [1, 5]. The gut microbiota forms a barrier against pathogens, maintaining colonization resistance of the host. The well adapted bacteria have developed this capacity through a continuous interaction with the human host under strong selection pressure. The gut microbiota can also activate the immune, endocrine and nervous systems of the host, including the brain. The immunomodulatory role of the microbiota is largely determined by the presence of bifidobacteria and lactobacilli [1, 6]. Residents of the gut flora produce bioactive substances, such as vitamins and hormones, participate in the metabolism of proteins, lipids, carbohydrates and nucleic acids, and regulate the composition of the intestinal gas [4].

Imbalances in the gut flora called dysbiosis are characterized by the increased abundance of potentially pathogenic microorganisms (pathobionts) [2, 7] and can lead to metabolic diseases, inflammation and visceral pain or induce changes in the central nervous system (CNS), which, in turn, can cause behavioral and cognitive disorders [2, 8–10]. Shifts in the microbiota composition were shown to accompany obesity, allergy, type 2 diabetes, cardiovascular and autoimmune diseases [2, 11]. The range of factors that negatively affect the composition and functions of the microbiota is continuously expanding; among them are environmental pollution (life in a megalopolis) [12], poor diet (the growing popularity of fast food) [13], and stress [14]. It is also known that dysbiosis can result from antibacterial therapies that kill the native microbial flora and therefore stimulate the unnatural selection of antibiotic-resistant pathogens [1].

A symbiotic relationship with bacteria is very important for the host. Highly responsive to a variety of factors, the microbial population of the GIT is a sensitive indicator of the host's health [1, 4]. Therefore, the microbiota could serve as a potential therapeutic target for the treatment/management of different pathologies, including dysbiotic conditions. Various approaches and strategies are used to regulate the human microbiome: diets, intake of pro- and prebiotics, and surgical procedures. Some authors indicate that physical activity has a positive effect on the microbiota when combined with a balanced diet [15, 16]. In this work we propose the use of selective inhibitors that target certain bacterial genera or species and therefore suppress bacterial growth, reduce virulence of pathogenic and opportunistic microbes and contribute to the restoration of the taxonomic balance of the gut microbiota. Eukaryotic type serine/threonine protein kinases (STPKs) can serve as a target for such inhibitors.

Classification of STPKs of gram-positive bacteria

Phosphorylation and dephosphorylation of proteins are the major molecular mechanisms that regulate processes inside the cell. An average bacterial phosphoproteome contains about a hundred of phosphorylation sites. An exception here is the phosphoproteome of *Mycobacterium tuberculosis* with about 500 sites [17]. For eukaryotic proteomes, the number of phosphorylation sites is an order of magnitude higher [18].

Serine/threonine protein kinases of both bacteria and human are an essential component of signal transduction [19, 20]. Eukaryotic type STPKs were first identified in bacteria [21–25] about 20 years ago. To date, we know that they are involved in the regulation of cell division and growth [26–28], biofilm formation [29], response to oxidative stress [30], and sporulation [31]. STPKs play an important role in the development of virulence and pathogenicity in bacteria [21, 32, 33]. They also participate in the pathogenesis of microbial diseases caused by *M. tuberculosis*, *Streptococcus pneumoniae*, *Staphylococcus aureus*, and other microorganisms [20, 25].

STPK inhibitors are ATP-competitive. Therefore, it appears tempting to employ selective inhibition of STPKs of pathogenic and opportunistic microorganisms by ATP-competitive inhibitors for the restoration of the compositional and functional balance of the human microbiota

Previously, we proposed a classification of eukaryotic type STPKs of gram-positive and gram-negative bacteria [19]. We analyzed a number of microbial STPKs found in pathogenic and non-pathogenic *Mycobacterium*, *Staphylococcus*, *Actinomyces* (and some others) and in probiotic members of the symbiotic human microbiota, namely *Bifidobacterium* and *Lactobacillus*. Our classification was based on the physiochemical properties of 9 amino acid residues that line the surface of the adenine-binding pocket. The amino acid residues were selected based on the visual analysis of 3D structures of bacterial STPKs available in the Protein Data Bank [34]. Amino acid sequences of bacterial STPKs were analyzed in parallel; we also performed multiple alignment of all sequences of the studied bacterial protein kinases [19].

The analysis of STPKs' structures identified amino acid residues that interact with ATP. We were particularly interested in the residues of the adenine-binding pocket, since this region is more variable than phosphate- and ribose-binding sites. Conserved residues and residues that contribute to ATP binding only with their backbone atoms were excluded from consideration, since they were of no practical value for our classification. While analyzing the STPKs of gram-positive bacteria, we disregarded highly conserved amino acid residues typical for eukaryotic and bacterial protein kinases, but instead focused on the substitutions in the positions that are conserved in eukaryotes and do not affect the functional activity of bacterial kinases. Of particular interest was catalytic subdomain V (STPKs have 12 conserved subdomains that are typical for both eukaryotic and bacterial STPK [35, 36]). Compared to other subdomains, the V-subdomain of eukaryotic protein kinases is quite variable and does not contain any conserved motif. However, this domain harbors amino acid residues that constitute the hinge region linking 2 lobes of a catalytic domain. By changing orientation of the catalytic and protein-binding regions, STPK activity can be regulated. Therefore, the V-subdomain, or, to be more precise, the hinge sequence can be a basis for STPK classification.

To sum up, we visually analyzed the structures of STPKs and their amino acid sequences and selected 9 variable amino acid residues whose side chains are exposed into the inside of the adenine-binding pocket: Leu17, Val25, Ala38, Val72, Met92, Tyr94, Val95, Met145, and Met155 (the residues are numbered according to the PknB sequence of *M. tuberculosis*) (Fig. 1).

The residues we selected constitute a signature sequence for the adenine-binding pocket. Conservation of the studied signature residues varied. Based on the analysis of the signature formed by 9 amino acid residues, we drafted a classification of STPKs of gram-positive bacteria. According

to the proposed classification, all studied kinases were organized into 20 groups (Fig. 2). Of note, the main criterion for grouping was the presence of a specific combination of a hydrogen bond donor/acceptor and aromatic residues in specific positions of the adenine-bonding pocket signature. Of 20 groups, 13 were species-specific. A few of the remaining 7 groups were characteristic of pathogens only [19]. Thus, the STPK classification allowed us to organize the studied bacterial genera and species into groups. In each group the configuration of the adenine-binding site (shape, volume, and depth) was specific, therefore we hypothesized that selective inhibitors targeting kinases from one group will not interact with (or will weakly interact with) kinases from other groups. In this light, our classification may find its practical application in the development of ATP-competitive inhibitors of bacterial eukaryotic-type STPKs.

Practical application of the proposed classification: selective inhibitors of STPKs

The composition of the gut microbiota is being actively studied. According to preliminary estimates, the large intestine is home to over 400 bacterial species that belong to a few taxonomic groups [37, 38]. As a rule, the gut microbiota of an adult human is dominated by *Firmicutes*, *Bacteroides*, *Actinobacteria* and *Proteobacteria* [39]. The microbial community seems to be unique for each individual [37, 40, 41] and can undergo significant changes due to exposure to various internal and external factors.

In dysbiosis, the abundance of probiotic flora diminishes and pathogenic bacteria start to grow leading to pathology [5]. So far, the association has been established between the GIT microbiota and a number of diseases. Morgan et al. have found a correlation between imbalances in the gut microbiota composition and ulcerative colitis and Crohn's disease [42]. While the gut microbiota of a healthy individual is dominated by 4 bacterial phyla [37, 43], the microbiota of patients with ulcerative colitis and Crohn's disease is characterized by lower taxonomic diversity and reduced abundance of *Firmicutes*. Besides, in Crohn's disease the abundance of *Clostridia* also changes [42, 44]. Reduced taxonomic diversity of the gut microbiota was also demonstrated in patients with psoriasis. Compositional shifts in the microbiota of psoriatic patients were associated with the increased abundance of 4 bacterial genera, including *Corynebacterium*, *Staphylococcus*, and *Streptococcus* [45].

The literature extensively reports on the association between different metabolic disorders and compositional changes in bacterial populations inhabiting the GIT. For example, Larsen et al. estimated differences between the gut microbiota of patients with type 2 diabetes and healthy individuals [46], revealing significantly lower abundances of *Firmicutes* and *Clostridia* and an increased abundance of *Lactobacillus* in diabetics as compared to healthy individuals [46]. Lactobacilli constitute a heterogenic group of microorganisms with immunomodulatory properties [47]. These properties seem to be a prerequisite of inflammation in patients with diabetes mellitus [46]. Tana et al. discovered that *Lactobacilli* were more abundant in patients with irritable bowel syndrome (IBS) than in the controls [48]. However, the presence of certain strains of *Lactobacillus* is very beneficial for patients with Crohn's disease and ulcerative colitis. The analysis of inflamed mucosa samples collected from such patients revealed the presence of some microorganisms (including *Lactobacillus* and *Bifidobacterium*) that can actually protect the intestinal mucosa from inflammation. The explanation here is that some *Lactobacillus* strains, in particular *L. casei* and *L. plantarum*, inhibit expression of key proinflammatory cytokines and chemokines and neutralize proinflammatory effects of *Escherichia coli* [49].

Considering the above, our classification comes handy when there is a need to restore a normal taxonomic composition of the microbiota; this can be achieved by the selective targeting of certain groups of bacteria: *Lactobacillus* (XIII) [19] in patients with type 2 diabetes or IBS, and *Corynebacterium*, *Staphylococcus* and *Streptococcus* (IV, X) in psoriatic patients.

Besides, the majority of STPKs are key regulators of bacterial growth: their inhibition can result in suppressed microbial growth. Selective inhibition of STPKs, including the STPKs of symbionts, can alter the composition of the microbiota without killing the indigenous microbes, though their growth and activity will be slightly affected. A good example here is growth inhibition of opportunistic *Actinomyces* and *Corynebacterium* belonging to groups III and IV, according to our classification [19]. Thus, a good STPK inhibitor can correct the composition of the human microbiota.

Because of the structural similarity of ATP-binding sites in human and bacteria, there is a chance of unintended inhibition of human protein kinases. Although catalytic domains of bacterial and human kinases are only 30 % identical, human protein kinases should be taken into account when developing selective inhibitors of bacterial STPKs. The human kinome encompasses 518 protein kinases. Using multiple alignment, we established that 324 of them contain Hanks subdomains usually found in eukaryotic STPKs. To distribute human kinases into groups according to our classification scheme, we identified 9 signature amino acids in the selected 324 kinases. It turned out that human kinases can be attributed to only 4 groups: I, II, VIII, and XII (Fig. 3). Of all analyzed human protein kinases, only 8.6 % ended up in "bacterial" groups. It should be noted that inhibition of unclassified human protein kinases by the compounds targeting classified STPKs is unlikely due to certain differences in their binding sites.

The need may arise to obtain selective inhibitors of STPKs of pathogens. Such inhibitors must be selective towards both human protein kinases and STPKs of microbial symbionts. Therefore, three clusters should be formed based on the groups that contain STPKs of pathogens, STPKs of symbionts; these

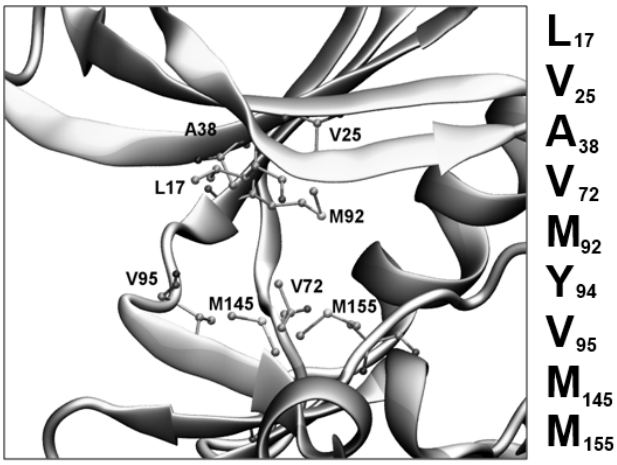

Fig. 1. A simplified representation of the adenine-binding pocket showing the signature amino acid residues (Tyr94 is not shown). The first three residues belong to the glycine loop (residues 17–38). They form the "ceiling" of the adenine-binding pocket. The hinge sequence consists of residues 92–96 (Met92 is the gatekeeper), but residues 93 and 96 contribute to ligand binding only with their backbone atoms, therefore, they were excluded from consideration

clusters should be screened for pathogen-specific kinases. For this purpose, we built a Venn diagram (Fig. 3).

Fig. 3 shows that kinases belonging to groups VII, X, XVIII, and XIX (see the Table) should be considered when developing selective inhibitors of STPKs found in pathogens. These groups include only kinases of pathogens, and the inhibitors targeting these groups are very unlikely to interact with human protein kinases or STPKs of symbiotic bacteria.

Groups VII and XIX include STPKs of *M. tuberculosis*, namely PknK and PknI. PknK is involved in the translation control at different stages of bacterial growth, contributes to adaptation and pathogenicity mechanisms in mycobacteria [50, 51]. It was demonstrated experimentally that *pknK* expression is higher in the virulent strain H37Rv of *M. tuberculosis* than in the avirulent strain H37Ra [50]. PknI has strong homology to Stk1 of *Streptococcus agalactiae* promoting virulence [52].

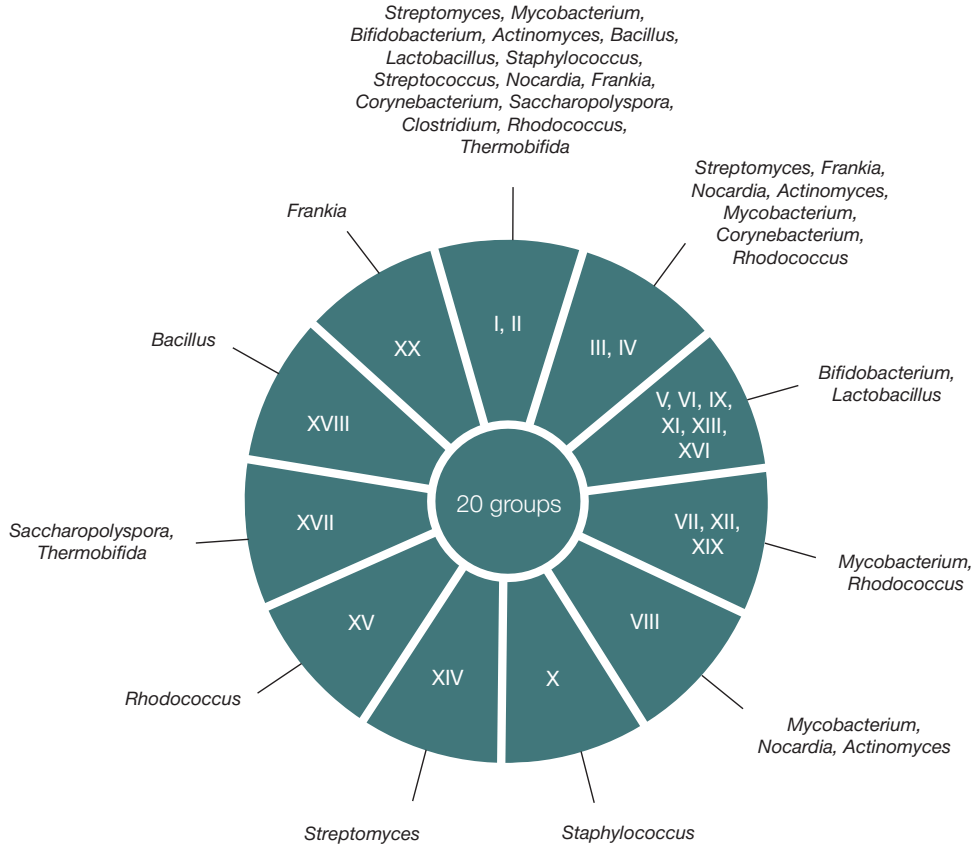

Fig. 2. Groups of STPKs of gram-positive bacteria (Zakharevich et al., [19])

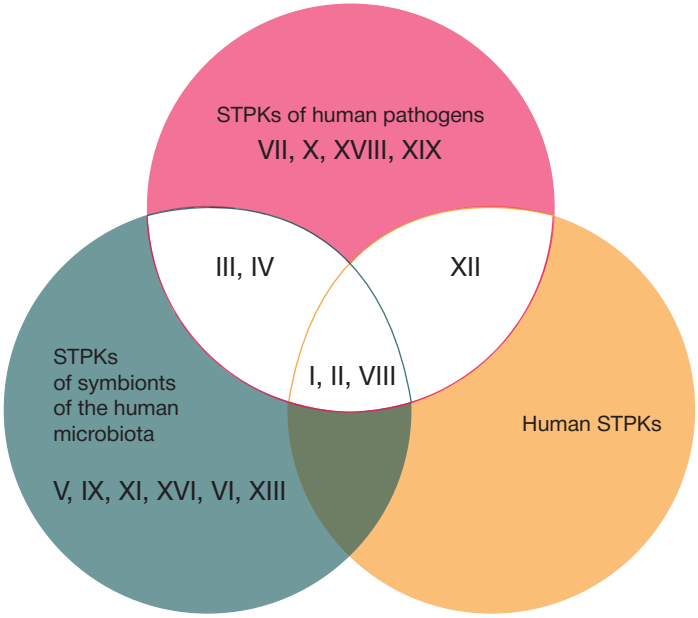

Fig. 3. This Venn diagram represents distribution of groups of serine/threonine protein kinases into three clusters. Roman numerals represent kinase groups according to our classification (Zakharevich et al., [19]). Groups XIV, XV, XVII, and XX are not included because: 1) the microorganisms belonging to these groups are neither pathogens nor symbionts of the human microbiota and 2) no similarities were detected between STPK signatures of these 4 groups and signatures of human protein kinases

The profile of STPKs from groups VII, X, XVIII, and XIX, characteristic for pathogens

Group	STPK	Signature	Genus (species)	Pathology
VII, XIX	PknK, PknI	[IV]VAVMYHLT, LSVVMYIVK	*Mycobacterium* *(M. tuberculosis, M. bovis, M. smegmatis, M. marinum, M. ulcerans)*	Tuberculosis, Buruli ulcer, Fish tank granuloma, opportunistic infections
X	Stk (PknB)	LVAVMYILF	*Staphylococcus* (*S. aureus, S. haemolyticus, S. epidermidis*)	Endocarditis, sepsis, peritonitis, abscesses, skin infections
XVIII	YbdM	ITVPMYML[IV]	*Bacillus* *(B. weihenstephanensis, B. cytotoxicus)*	Food poisoning (diarrhea)

Having said that, we assume that inhibition of these kinases will suppress *M. tuberculosis* growth and diminish pathogenicity and virulence of its strains.

Group X includes STPKs of *Staphylococcus*. Staphylococcal STPKs modulate the cell wall structure and are involved in promoting bacterial virulence [53]. Consequently, inhibition of kinases belonging to this group will reduce virulence of *Staphylococcus*.

Group XVIII is represented by the YbdM protein kinase of *Bacillus* that phosphorylates the two-component system DegS/U. In turn, this system affects biofilm formation, formation of complex colonies and microbial motility [54]. Therefore, selective inhibitors of STPKs of this group will disrupt the above mentioned processes.

Human protein kinases whose signatures coincided with the signatures of bacterial protein kinases belong to the following kinase classes: AGC[1], CAMK[2], CMGC[3], STE[4], and also to the families of yet unclassified protein kinases, such as IKK and NEK. The role of these protein kinases in the human body is varied. For example, kinases of the ROCK family participate in the Rho-induced formation of actin stress fibers and focal adhesion formation, as well as in platelet activation, smooth muscle contraction, neutrophil chemotaxis, etc. [55]. PAK kinases phosphorylate some cytoskeletal proteins and regulate their activity. Some authors indicate their role in the regulation of MAPK signal pathways in mammalian cells [56, 57]. Kinases of the NDR family were shown to have a role in embryonic development, neurological processes and cancer mechanisms [58]. However, it does not mean that inhibition of these human kinases should be avoided in every single case. For example, the increased expression of ROCK kinases is associated with a number of disorders (bladder cancer, breast carcinoma, etc.), and PAK1 kinase is associated with joint diseases (osteoarthritis, rheumatoid arthritis) because activation of certain signaling cascades in which this kinase is a major mediator leads to the increased expression of marker genes linked to osteoarthritis [59].

Thus, regulation of the taxonomic composition of the human microbiota is a complex task and requires a high level of expertise in different research fields. A good knowledge of microbial genetics and processes inside the human cells is a must. Bioinformatics and chemistry will also be involved, as huge data arrays will have to be analyzed and 3D structures built of the members of at least major kinase groups.

CONCLUSIONS

Recently researchers have come to realize that the human microbiota is crucial for human health. The most diverse and large population of microorganisms inhabits the gastrointestinal tract. Its residents have been co-evolving with human. Taxonomic imbalances in the gut microbiota can lead to pathology, including ulcerative colitis, Crohn's disease, diabetes, etc.

Approaches to restoring the taxonomic composition of the microbiota should consider the effect and selective capacity of the compounds intended to inhibit microbial growth. It is important that correction of taxonomic imbalances should target both pathogens and symbionts. Therefore, the effect of kinase inhibitors on the gut flora should be strong enough to suppress the growth of pathogens and reduce their virulence and at the same time gentle enough to only minimally affect human protein kinases. Selective inhibitors of STPKs seem to be ideal for this purpose.

An idea of using target STPK inhibitors is not novel [20, 60, 61]. In our previous work we compared human kinases and kinases of pathogens (*Plasmodium*, *Trypanosoma*, *Leishmania*) and showed that in spite of quite conserved structure of the catalytic domain and binding sites in particular, creation of selective inhibitors is possible [62]. The proposed classification of STPKs is the first step towards developing effective selective inhibitors of bacterial protein kinases.

We propose the following algorithm: (1) a comparative analysis of taxonomic composition of microbiotas in healthy and diseased individuals and identification of those bacterial groups (species or genera) that are overabundant in pathology; (2) the analysis of groups that include STPKs of the identified microbes; (3) the *in silico* search of inhibitors (low molecular weight compounds) of protein kinases belonging to the identified groups (modeling of 3D structures, molecular docking); (4) tests of the obtained compounds. Steps (2) and (3) imply consideration of: functions of target STPKs; non-target STPKs bacteria included into the selected groups; 3D structure(s) of a typical protein kinase representative from each group to expedite selection of good inhibitors.

[1] AGC — a class of protein kinases whose activity is regulated by cyclic GMP/AMP. This class includes the so-called protein kinases C; their activity can be regulated by diacylglycerol, phospholipids and calcium ions.

[2] CAMK — calcium/calmodulin-dependent protein kinases.

[3] CMGC — a class of protein kinases consisting of cyclin-dependent protein kinases (C), the so-called MAP-kinases (M) and enzymes that can phosphorylatie glycogen synthase (G).

[4] STE — serine/threonine kinases first identified in yeasts.

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METAGENOMIC LIBRARY PREPARATION FOR ILLUMINA PLATFORM

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Metagenomic sequencing is widely used in both scientific research and clinical practice for characterization of taxonomic profiles including estimation of relative abundance of prokaryotes in microbial communities in various media. Metagenomic sequencing of single marker genes is an excellent tool for studying the human microbiome. Unlike whole-genome sequencing, it targets those genome regions that can be instrumental in identification of microorganism species and genus. The 16S ribosomal RNA (16S rRNA) gene sequence is highly conserved but at the same time there are regions containing species-specific sequences that can discriminate between different bacteria and archaea. These regions can be amplified using universal primers, which makes the whole procedure more cost-effective and less time-consuming. Good primers and protocol design for PCR at the step of library preparation is crucial for achieving high data accuracy. Below we describe how to choose the optimal PCR protocol and universal primers to amplify V3 and V4 regions of the 16S rRNA gene for further sample sequencing using Illumnia platform.

Keywords: microbiome, metagenomic sequencing, ribosomal RNA, 16S rRNA, polymerase chain reaction, universal primers, library preparation, double barcode

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ОСОБЕННОСТИ ПОДГОТОВКИ БИБЛИОТЕК ДЛЯ МЕТАГЕНОМНОГО СЕКВЕНИРОВАНИЯ ОБРАЗЦОВ НА ПЛАТФОРМЕ ILLUMINA

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Метагеномное секвенирование, позволяющее определять таксономическую принадлежность и долю прокариотических организмов в сообществах в разных средах, широко используется не только в научной деятельности, но и в медицинской практике. В настоящее время для изучения микробиома человека применяется так называемое метагеномное секвенирование маркерных генов, при котором секвенируется не весь геном, а лишь те его регионы, по которым можно установить родовую и иногда видовую принадлежность микроорганизмов. Чаще всего для амплификации выбирают участки гена 16S рибосомальной РНК (16S рРНК), последовательность которого, с одной стороны, высококонсервативна, а с другой — содержит вариабельные участки, которые отличаются однонуклеотидными заменами в случае с разными микроорганизмами. При этом для амплификации таких участков генома возможен подбор универсальных праймеров, что значительно снижает стоимость и время исследования. Точность секвенирования в этом случае обеспечивается точностью подбора универсальных праймеров и оптимальностью условий проведения ПЦР на этапе подготовки библиотеки для секвенирования. В статье мы описываем подход к подобру универсальных праймеров и условий ПЦР для амплификации регион-специфической части V3 и V4 участков гена 16S рРНК для дальнейшего секвенирования образцов на платформах Illumina.

Ключевые слова: микробиом, метагеномное секвенирование, рибосомальная РНК, 16S рРНК, полимеразная цепная реакция, универсальные праймеры, библиотеки для секвенирования, двойное баркодирование

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The human body is home to a number of bacterial communities inhabiting the mouth cavity, gut, genitourinary system, etc. [1]. The totality of microorganisms that have symbiotic relationships with the host is called the microbiome [1]. Study of the human microbiome provides understanding of what bacteria live in healthy and diseased individuals [2].

Sequencing of those regions of bacterial genomes that discriminate between bacterial species and sometimes genera is called metagenomic sequencing, or marker gene sequencing, and is currently widely used in microbiome studies [3]. Regions of the 16S ribosomal RNA (16S rRNA) gene are especially convenient for sequencing. This gene is highly conserved in

prokaryotes enabling the use of universal primers to amplify the target sequence, which makes the whole procedure cost-effective and not so time-consuming [4, 5]. The 16S rRNA gene harbors conserved and variable regions. The latter contain single-base substitutions that are instrumental in identifying microbial species or genera: once these substitutions are detected by sequencing, they can be matched against regularly updated public databases.

One of the core steps of metagenomic sequencing workflow is sample preparation, i.e. converting source nucleic acids into a library of DNA fragments ready to be loaded onto the sequencer. There are a lot of different sequencing platforms, and although their sample preparation strategies are more or less the same, there are some nuances related to the techniques employed for signal detection during sequencing [6]. Sample preparation aims at obtaining DNA fragments that serve to identify a species or a genus of the studied microorganism. In metagenomics accuracy is largely determined by a good choice of primers necessary to produce amplicons by polymerase chain reaction (PCR) [7, 8]. In 2013 Klindworth et al. compiled a list of 512 primer pairs organized into three subgroups based on the next generation sequencing (NGS) technique they can be used for: group 1 consisted of primers for Illumina and Ion Torrent platforms (small amplicons), group 2 consisted of primers for 454 Life Science platform (middle-sized amplicons), and primers included in group 3 were intended for PacBio and other similar platforms (large amplicons) and could be also used to prepare genomic libraries of colonial species [9]. Each group offers a few universal primer pairs for archaea and bacteria instrumental in species/genus identification.

In this work we discuss some aspects of metagenomic sample sequencing with Illumina with a particular focus on library preparation for further sequencing [10]. Illumina-based sequencing takes place in the flow cell coated with single-stranded oligos that are complementary to library adapters ligated to source DNA fragments and enable hybridization. Polymerase lengthens hybridized DNA fragments attached to the flow cell surface. PCR produces multiple copies of a single template molecule forming millions of dense clusters. Clusters are then sequenced in parallel: complementary strands are generated by fluorescently tagged nucleotides, and the emitted signal is recorded after the addition of each nucleotide to the strand. This technology sets certain requirements for sample preparation explained below.

Library preparation for further sequencing with Illumina

Sequencing can be performed on various types of biological material, such as saliva, ear wax, nasal mucosal swabs, etc. We focused on the general aspects of library preparation for sequencing with Illumina regardless of the sample type. Basically, the sequencing workflow includes 5 steps: 1) extracting the intact DNA from the sample; 2) selecting genome regions for sequencing and choosing primers for further PCR-based amplification (PCR quality is very important because it determines sequencing quality); 3) double barcoding of the obtained libraries; 4) sequencing itself; 5) bioinformatic analysis of the obtained data.

There are a lot of protocols and reagent kits for effective DNA extraction [11] depending on the type of the analyzed sample; therefore, there is no need to discuss this step in more detail here. In our study quality control of the extracted DNA was performed by agarose gel electrophoresis, concentration was measured by Qubit 3.0 Fluorometer (Thermo Fisher Scientific, USA) according to the manufacturer's protocol [12].

DNA extraction is followed by PCR amplification of the studied DNA fragments for further sequencing. For this work we chose a few regions of the 16S rRNA gene for the reasons explained above. Quality of the obtained fragments depends on the complementarity of the selected primers to the regions of the 16S rRNA gene [13, 14]. Primers consist of a region-specific sequence complementary to the flanking region of the target fragment and a synthetic sequence non-complementary to the region-specific sequence that will hybridize to the adapter. It is important that at least four 3′-end nucleotides should be non-complementary within and between primers to avoid primer-dimer formation. Even a small mismatch in complementarity of 3–4 nucleotides at the 3′-end of the primer can significantly reduce PCR quality even if the annealing temperature has been adjusted [15, 16].

There are a lot of regularly updated databases containing sequences of the 16S rRNA gene identified for a plethora of microbial species [17, 18], which facilitates rapid selection of universal primers using a special software if necessary [19]. For this work we chose universal primer pairs for the V3 and V4 regions of the 16S rRNA gene [23]. The synthetic sequence of the chosen primers was represented by sequences complementary to Nextera and Truseq adapters (Table 1).

Once the primers are selected, the PCR protocol needs to be optimized (a number of parameters have to be adjusted, such as primer concentration, DNA concentration, annealing temperature, Mg^{2+} concentration, number of cycles, etc.) to obtain a sufficient amount of good quality amplicons for further sequencing. PCR quality control is performed by agarose gel electroforesis. Negative and positive controls are a must. A negative control is usually a PCR mix without the DNA template. In our case, two DNA samples were used as positive controls: one of *Rhizobium* and another of *Rhodoccocus* bacterial genera.

PCR yield can be affected by primer-dimer formation [20]. Primer dimers also occur when performing PCR quality control in the agarose gel (Fig. 1). Diluting primers or adjusting the annealing temperature can be a solution. The optimal annealing temperature determines the purity of the reaction product since it facilitates primer attachment to DNA. State-of-art equipment makes it possible to do a temperature gradient to optimize the annealing temperature in a single run. High yields also depend on Mg^{2+} levels: Mg^{2+} ions bind to dNTP,

Table 1. Universal primer pairs consist of a sequence complementary to the V3 and V4 regions of the 16S rRNA gene and a synthetic sequence complementary to Nextera and Truseq adapters

Adapter	Primer sequence	
Nextera	forward	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG
	reverse	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC
Truseq	forward	ACACTCTTTCCCTACACGACGCTCTTCCGATCTCCTACGGGNGGCWGCAG
	reverse	GTGACTGGAGTTCAGACGTTGGCTCTTCCGATCTGACTACHVGGGTATCTAATCC

primers, DNA template and chelating agents (EDTA) present in the buffer [21]. Polymerase activity is known to grow at high Mg^{2+} concentrations, though polymerase specificity thereby decreases. As a rule, a range of Mg^{2+} concentrations from 1 to 4 mM with a 0.5 mM dilution step is tested to select the optimal concentration for the reaction mix.

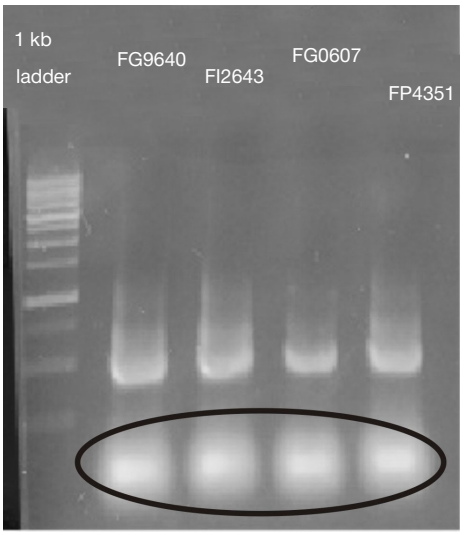

Fig. 1. Agarose gel with PCR products after electrophoresis. The first well contains a DNA-size marker, the rest contain the samples. Primer dimers (PCR by-products) are marked with a black oval

When optimizing the PCR protocol, we found out that primers complementary to Nextera adapters were the best for the amplification of the V3 and V4 regions of the 16S rRNA gene. It is probably because the unique Nextera sequence is non-complementary to the regions of the studied bacterial genome, which prevents formation of side products. The optimized PCR protocol is shown in Table 2. PCR was performed using Step One Plus system (Applied Biosystems, USA).

The harvested libraries are dual-indexed (barcoded) in another PCR step. Barcoding is adding an index sequence of 8 nucleotides to the DNA fragment to facilitate further discrimination between different sample sets [22]. There is a wide selection of reagent mixes for barcoding that can be used in Illumina-based sequencing, such as Nextera XT Index kit. We used oligos synthesized by Evrogen, Russia. We ran a few tests with various PCR parameters to discover that barcoding yields did not depend on the purity of the DNA template and required no sample purification. The optimal PCR parameters for Nextera-based barcoding with Nextera primers (Table 3) are shown in Table 4.

It should be reminded that poor library quality control will entail mistakes during sequencing. We usually perform quality control using Agilent Bioanalyzer 2100 (Agilent Technologies, USA) (Fig. 2).

Table 2. Parameters of PCR for amplification of the V3 and V4 regions using universal primers for the Nextera adapter

Composition	V, µl	Protocol		
		T, °C	t, s	Number of cycles
Forward primer (C = 1 µM)	5,0	95	180	1
Reverse primer (C = 1 µM)	5,0	95	30	25
Taq-polymerase	0,5	55	30	
dNTP	1,5	72	30	
PCR buffer	2,5			
DNA	2,5	72	300	1
mQ	8,0			

Table 3. Nextera index primers

Primer name	Primer sequence
i701	CAAGCAGAAGACGGCATACGAGATTCGCCTTAGTCTCGTGGGCTCGG
i702	CAAGCAGAAGACGGCATACGAGATCTAGTACGGTCTCGTGGGCTCGG
i703	CAAGCAGAAGACGGCATACGAGATTTCTGCCTGTCTCGTGGGCTCGG
i704	CAAGCAGAAGACGGCATACGAGATGCTCAGGAGTCTCGTGGGCTCGG
i501	AATGATACGGCGACCACCGAGATCTACACTAGATCGCTCGTCGGCAGCGTC
i502	AATGATACGGCGACCACCGAGATCTACACCTCTCTATTCGTCGGCAGCGTC
i503	AATGATACGGCGACCACCGAGATCTACACTATCCTCTTCGTCGGCAGCGTC
i504	AATGATACGGCGACCACCGAGATCTACACAGAGTAGATCGTCGGCAGCGTC

Table 4. Parameters of PCR for barcoding with Nextera primers

Composition	V, µ	Protocol		
		T, °C	t, s	Number of cycles
Index 1 (C = 1 µM)	5,0	95	180	1
Index 2 (C = 1 µM)	5,0	95	30	10
Taq-polymerase	0,5	55	30	
dNTP	1,5	72	30	
PCR-buffer	2,5			
1st PCR amplicons	5,0	72	300	1
mQ	5,5			

Sample sequencing

We will not focus on the sequencing step itself in this article, because there are standard sequencing protocols supplied by the vendor [24, 25]. We performed MiSeq paired-end sequencing (Illumina) with 250 b. p. reads according to the standard protocol.

Bioinformatic analysis

The obtained nucleotide sequences are processed and classified as suggested by the Ribosomal Database Project, ver. 11.5 (Michigan University, USA), using the RDPTools ver. 2016-07-21 [26, 27]. Classification confidence threshold (-conf) should be set to 50 % as recommended by [28].

Quality control is essential in sequencing. At least 95 % of sequences in each sample must be high quality, i. e. contain no adapters or contaminating elements that cannot be mapped onto the human genome. The number of reads per sample is especially important in metagenomic sequencing. But on the whole, there is no universal rule here and the number of reads depends mostly on the purpose of the study. If the study aims at identifying dominant bacteria in the sample, the number of reads can be low. For example, 350 reads per each of 22 human gut samples revealed the presence of two dominating bacteria: *Firmicutes* (75 %) and *Bacteroidetes* (18 %) [29]. However, higher read numbers increase chances of discovering microbial "minorities" in the sample and reduces effects of the sampling error. A high-resolution metagenomic analysis requires at least 10,000 reads [29].

The proportion of unclassified sequences and unknown bacterial sequences, the median proportion of sequences for which both genus and species have been reliably identified should be consistent with the results of the 16S rRNA-based microbial metagenomic analysis. The proportion of unclassified sequences should not exceed 20 %, the genus is expected to be identified for at least 70 % sequences, while the species — for at least 50 %. Still, these figures may vary depending on the study.

Normally, a table is compiled based on the analysis, showing a taxonomic hierarchy (domain, phylum, class, etc.) and providing information on the taxonomic tree and relative abundance of taxa in the sample. An example of such table is Table 5.

CONCLUSIONS

Selecting primers for amplification is an important step in metagenomic sequencing of 16S rRNA gene regions. Quality of the obtained amplicons determines accuracy of sequencing. We suggest an approach to designing an optimal PCR protocol for sample preparation that can be used to adjust PCR parameters for library preparation and identified problems that may occur at this step. Although library preparation for metagenomic sequencing has been widely discussed in the literature [30], we have made an attempt to design a well-defined protocol, proposed optimal parameters for the amplification of 16S rRNA V3 and V4 regions using universal primers for further sequencing with Illumina. Of note, the quality of amplification yields depends on many factors, including the purity of the reagents, therefore our PCR protocol is not universal and its PCR conditions may vary depending on the reagents used.

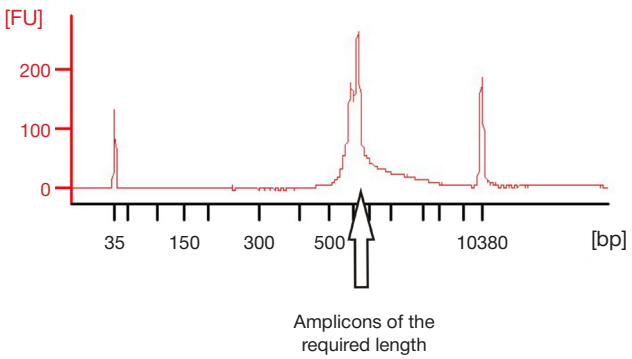

Fig. 2. Sequencing quality control with Agilent Bioanalyzer 2100

Table 5. Bioinformatic analysis: representation of results

Rank	*Name*	*rootrank*	*domain*	*phylum*	*class*	*order*	*family*	*genus*	Sample 1	Sample 2	Sample ...
1_domain	*Archaea*	Root	*Archaea*								
2_phylum	*Crenarchaeota*	Root	*Archaea*	*Crenarchaeota*							
3_class	*Thermoprotei*	Root	*Archaea*	*Crenarchaeota*	*Thermoprotei*						
4_order	*Acidilobales*	Root	*Archaea*	*Crenarchaeota*	*Thermoprotei*	*Acidilobales*					
5_family	*Acidilobaceae*	Root	*Archaea*	*Crenarchaeota*	*Thermoprotei*	*Acidilobales*	*Acidilobaceae*				
6_genus	*Acidilobus*	Root	*Archaea*	*Crenarchaeota*	*Thermoprotei*	*Acidilobales*	*Acidilobaceae*	*Acidilobus*			

Note. Rank refers to the elements of the taxonomic hierarchy; Name referes to the name of the taxon. These two tables are used for data filtering. For example, using the Rank column as a filter, one can retrieve information on the species/phyla only, etc. The next 7 columns (rootrank, domain, phylum, class, order, family, genus) provide information about the full hierarchal path. The last columns contain data on the relative abundance of the taxon in the studied sample. Sample names are specified in column titles

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A COMPARATIVE ANALYSIS OF SEMINAL AND VAGINAL MICROBIOTA OF MARRIED COUPLES BY REAL-TIME PCR WITH ANDROFLOR AND FEMOFLOR REAGENT KITS

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Many sexually transmitted diseases are caused by bacteria. While we fairly well understand the role of some microorganisms in the development of genitourinary tract infections, there is still a vast majority of those whose contribution is unclear. It is believed that sexual partners share their genitourinary microbiota, meaning that treatment regimens should be the same for both of them. This article reports results of the study of seminal and cervical microbial communities conducted in 50 married couples who did not use barrier birth control and did not take any antibiotics at least 3 months before the study. All couples presented with complaints of primary or secondary infertility, recurrent miscarriages or sought preconceptional counseling. The mean age of male and female participants was 34.8 ± 7.8 and 30.4 ± 6.2 years, respectively. Samples of the seminal fluid and vaginal secretions were studied by real-time polymerase chain reaction (real-time PCR) with Androflor and Femoflor reagent kits. The following bacteria were more frequent in the vaginal microbiota than in the seminal fluid: *Lactobacillus spp.* ($p < 0.005$), *Eubacterium spp.* ($p = 0.002$), *Gardnerella vaginalis* ($p = 0.002$), *Megasphaera spp./Velionella spp./Dialister spp.* ($p = 0.004$). *Ureaplasma spp.* was 3 times more frequent in women, *Mycoplasma hominis* was 4 times more frequent in men; however, this difference was not significant. In 4 (8 %) couples both partners had normal microbiota; 23 (46 %) couples shared at least one microbiota resident. Also, microbial communities were totally different in 23 couples. The obtained data indicate that both sexual partners should be examined to decide on the most effective treatment for each of them. Qualitative and quantitative real-time PCR assays Androflor and Femoflor provide comprehensive data essential for adequate treatment planning.

Keywords: seminal fluid, cervical canal, genitourinary tract, married couple, microbiota, microbial community, polymerase chain reaction, real-time PCR, Androflor, Femoflor

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СРАВНИТЕЛЬНЫЙ АНАЛИЗ БИОТОПА ЭЯКУЛЯТА И ЦЕРВИКАЛЬНОГО КАНАЛА МЕТОДОМ ПЦР-РВ С ТЕСТАМИ «АНДРОФЛОР» И «ФЕМОФЛОР» В СУПРУЖЕСКИХ ПАРАХ

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Многие инфекции, передающиеся половым путем, вызываются бактериями. Роль ряда микроорганизмов в этиологии урогенитальных инфекций хорошо известна, однако далеко не всех. Во врачебном сообществе также принято считать, что микробиота урогенитального тракта у половых партнеров одинакова, в связи с чем им следует назначать одинаковое лечение. В данной статье сообщается о результатах исследования биотопа эякулята и цервикального канала в 50 супружеских парах, живших без барьерной контрацепции и приема антибактериальных препаратов не менее чем 3 месяца до участия в исследовании. Все пары обратились к врачу по поводу первичного или вторичного бесплодия, привычного невынашивания беременности или предгравидарной подготовки. Средний возраст мужчин составил 34,8 ± 7,8 лет, женщин — 30,4 ± 6,2 лет. Эякулят и отделяемое цервикального канала исследовали методом полимеразной цепной реакции в режиме «реального времени» (ПЦР-РВ) с использованием тестов «Андрофлор» (у мужчин) и «Фемофлор» (у женщин). В биотопе цервикального канала достоверно чаще, чем в биотопе эякулята, встречались *Lactobacillus spp*. ($p < 0,005$), *Eubacterium spp*. ($p = 0,002$), *Gardnerella vaginalis* ($p = 0,002$), *Megasphaera spp./Velionella spp./Dialister spp*. ($p = 0,004$). *Ureaplasma spp*. в 3 раза чаще встречалась у женщин, а *Mycoplasma hominis* — в 4 раза чаще у мужчин, но различия статистически недостоверны. В 4 (8 %) супружеских парах у обоих партнеров была абсолютно нормальная микрофлора эякулята и цервикального канала; в 23 (46 %) парах были выявлены совпадения между биотопами по одному и более микроорганизмам; также в 23 парах биотопы полностью не совпадали. Полученные данные указывают на необходимость обследовать обоих партнеров в паре для выбора эффективного лечения каждого из них. Метод ПЦР-РВ с тестами «Андрофлор» и «Фемофлор», являясь качественным и количественным, позволяет получить врачу все данные, необходимые для планирования терапии.

Ключевые слова: эякулят, цервикальный канал, урогенитальный тракт, супружеская пара, биотоп, микробиоценоз, микробиота, полимеразная цепная реакция, ПЦР-РВ, «Андрофлор», «Фемофлор»

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To date, over 30 bacterial infections are known to be transmitted through sexual contact [1, 2]. It has been shown that *Chlamydia trachomatis* and *Mycoplasma genitalium* promote inflammation in the genitourinary system of both men and women, but pathogenicity of *Mycoplasma hominis* and *Ureaplasma spp*. is still a matter of debate, which is why genitourinary mycoplasmas are actively studied across the world [1–9].

The meta-analysis of 4,712 academic articles published over the period from 1960 to 2013 provides convincing evidence of the contribution of the genitourinary inflammation in the mother to the development of pregnancy complications, including preterm delivery and early or late perinatal morbidity [4]. Sexually transmitted infections (STIs) compromise semen quality, induce formation of antisperm antibodies and are a major cause of urethroprostatitis and infertility [1–3, 5–8, 10]. In a study conducted in women who had suffered preterm delivery, 50 % of the participants tested positive for *Ureaplasma spp*. in the amniotic fluid; however, this bacteria did not appear as a monoculture, but were detected in associations with opportunistic microorganisms, including *Fusobacterium*, *Mycoplasma*, *Sneathia*, *Bacteroides*, *Prevotella*, *Streptococcus spp.*, *Staphylococcus spp.*, *Enterococcus spp.*, *Leptotrichia*, *Peptostreptococcus*, *Gardnerella*, *Citrobacter*, *Lactobacillus spp.*, *Escherichia*, and *Haemophilus* [9].

Polymerase chain reaction (PCR) is a qualitative and quantitative test widely used in the clinical routine for quick and reliable identification of infectious agents, though it cannot identify co-occurring opportunistic pathogens. As indicated in the modern clinical practice guidelines, it is important to determine pathogen concentrations in the sample, especially when running tests for *Ureaplasma spp*. and *M. hominis* [10–15]. According to the European Association of Urology, *Ureaplasma urealyticumin* concentrations above 10^3 CFU/ml are considered pathogenic; this value influences the choice of treatment [5]. According to Russian clinical practice guidelines, treatment is not recommended if concentrations of *Ureaplasma spp*. and/or *M. hominis* are lower than 10^4 CFU/ml and there are no clinical/laboratory-confirmed signs of inflammation in the genitourinary tract [11, 14, 15]. *Androflor* and *Femoflor* qualitative and quantitative real-time PCR-based assays for male and female patients, respectively, meet the requirements of a diagnostic PCR for STIs.

An opinion is shared by the medical community and the public that microbiotas of the genitourinary tract of both sexual partners are identical in composition. Once an infectious agent has been detected in the ejaculate or cervical canal secretions of one partner, both members of the couple start receiving identical treatment, since the doctor assumes that their genitourinary tracts are inhabited by the same microbes. As a result, one of the partners is left underexamined and is prescribed therapy for no good reason. In one of our studies, we bacteriologically examined semen and cervical secretions of 117 married couples who did not use any birth control, to find out that in 84 % of the analyzed samples the identified bacteria were different [16]. So far, we have failed to find reports on any real-time PCR-based comparative analysis of the genitourinary microbiotas of both sexual partners.

The aim of this study was to compare ejaculate and cervical microbiotas of sexual partners in married couples using real-time PCR-based assays *Androflor* and *Femoflor*.

METHODS

We initiated a prospective study of 50 married couples who presented at the clinic of Ivanovo State Medical Academy over the period from October, 2016 to March, 2017 with complaints of primary or secondary infertility, recurrent miscarriages or sought preconceptional counseling. All couples did not use barrier birth control and did not take antibacterial medications at least 3 months before sample collection. Mean age was 34.8 ± 7.8 years for males and 30.4 ± 6.2 years for females.

Before semen collection, male patients were asked to pass urine to empty the bladder, wash their hands and penis with soap and water, and dry the glans penis and foreskin with a sterile disposable towel. Semen samples were obtained by masturbation, placed into the sterile container and delivered

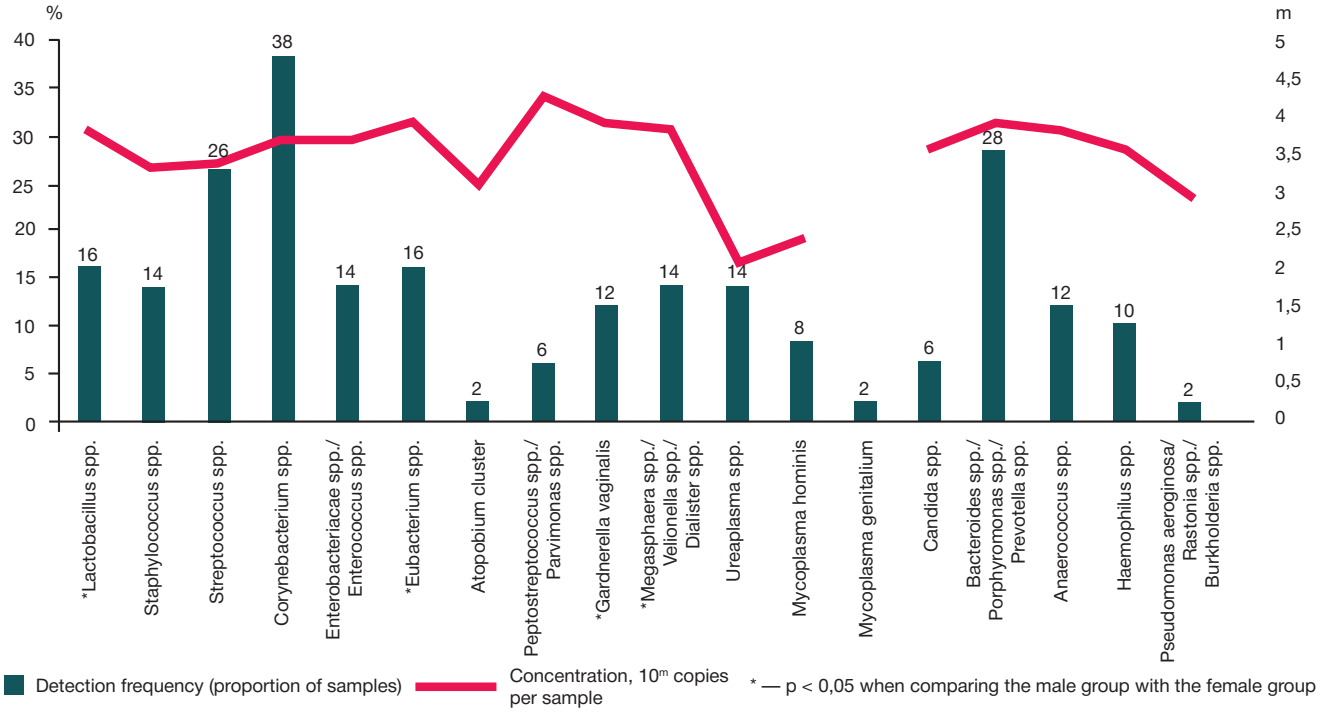

Fig. 1. Frequency (%) and average concentrations (copies per sample) of microorganisms in the semen. Data obtained with Androflor real-time PCR kit

to the lab within one hour after sample collection. In women, excess cervical mucus was removed with a cotton swab, and the cervix was washed with a sterile sodium chloride solution. A probe was inserted into the cervical canal to the depth of 0.5–1.5 cm; the probe was retrieved carefully to avoid contact with the vaginal walls. The participants abstained from sex for at least 3 days before sample collection.

All samples were analyzed using *Androflor* and *Femoflor* real-time PCR reagent kits (both by DNA-Technology TS, Russia) and the DT-96 PCR detection system (R&P DNA-Technology, Russia).

Statistical analysis was performed using Microsoft Excel 2013 and Statistica 10.0 (StatSoft, USA). Fisher's exact test was applied to determine statistical significance of the results. The difference was considered significant at $p < 0.005$.

RESULTS

Using *Androflor*, we detected no pathogens in the ejaculate of 16 (32 %) men; the semen of 7 participants (14 %) contained opportunistic *Staphylococcus spp.*, *Streptococcus spp*. and

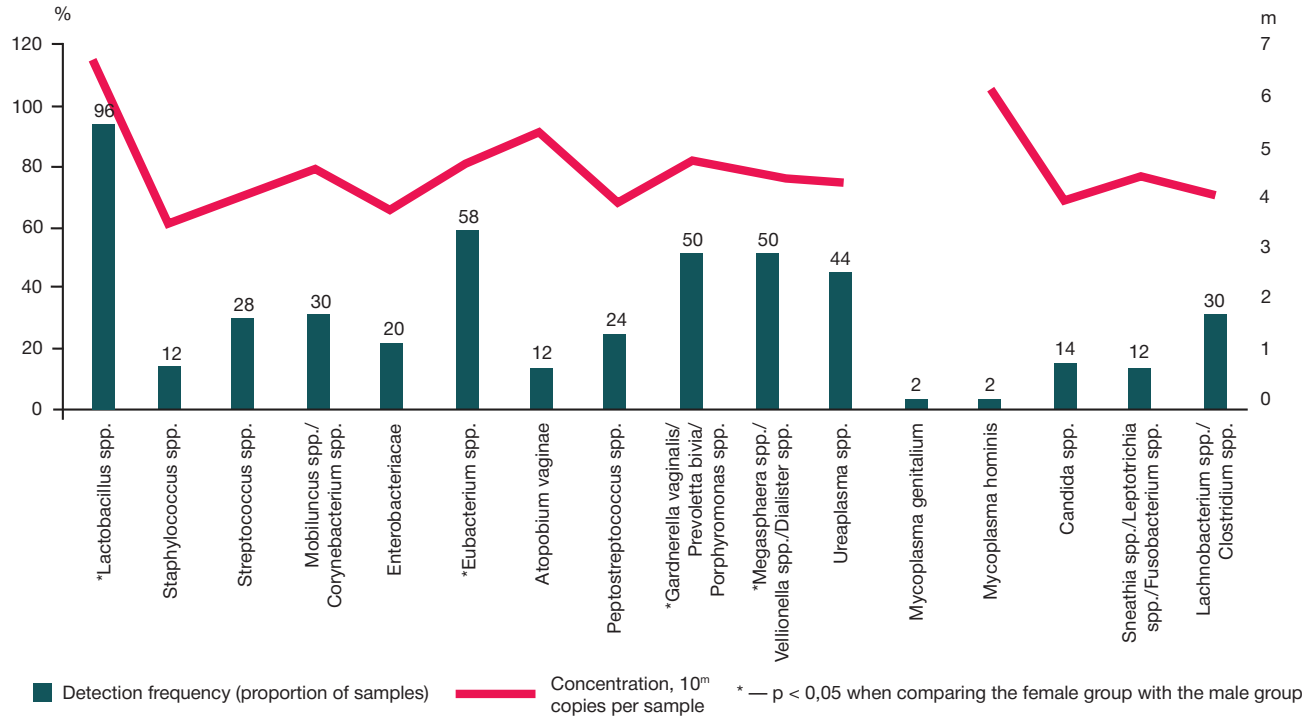

Fig. 2. Frequency (%) and average concentrations (copies per sample) of microorganisms in the cervical canal. Data obtained with Femoflor real-time PCR kit

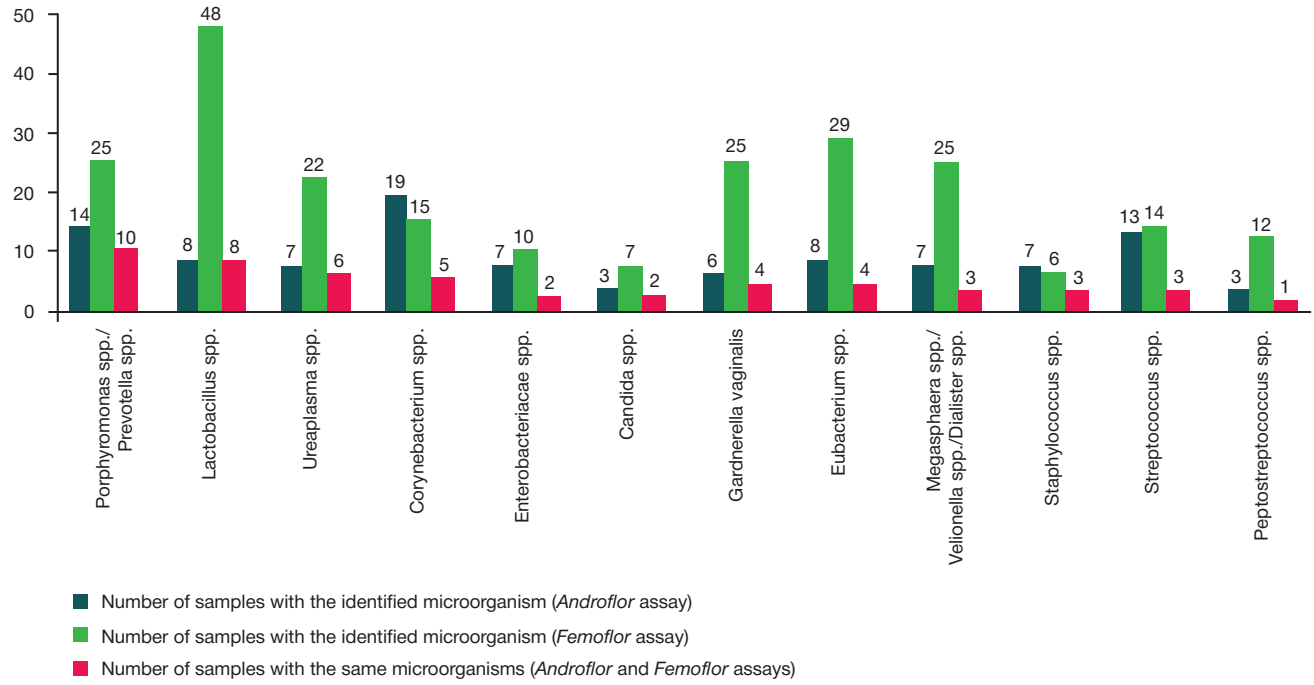

Fig. 3. Semen and cervical microbiotas with similar microbial composition

Corynebacterium spp. at non-pathogenic concentrations; the semen of 27 (54 %) men was dysbiotic. Using *Femoflor*, we detected only *Lactobacillus spp*. in the cervical secretions of 11 (22 %) women, indicating the absence of pathology; 25 (50 %) women had vaginal dysbiosis; the microbiotas of 14 (28 %) female participants were healthy, dominated by *Lactobacillus spp*. (> 80 %) and containing facultative anaerobes and obligate anaerobes in the absence of intracellular pathogens.

Microbial diversity of the semen and cervical secretions detected by *Androflor* and *Femoflor* is shown in Fig. 1 and Fig. 2, respectively. All women, except one, had *Lactobacillus spp*. in their cervical samples. The most common species detected in male samples was *Corynebacterium spp*. (38 %), a member of the healthy semen microbiota. Compared to the semen samples, the cervical samples showed significantly higher occurrence of *Lactobacillus spp*. ($p < 0.005$), *Eubacterium spp*. ($p = 0.002$), *Gardnerella vaginalis* ($p = 0.002$), and *Megasphaera spp./Velionella spp./Dialister spp*. ($p = 0.004$). No significant differences were observed regarding other species, but *Ureaplasma spp*. was 3 times more frequent in female cervical secretions than in the semen (22 vs. 7 samples, respectively); *Mycoplasma hominis* was 4 times more frequent in men than in women (4 vs. 1 sample, respectively). One female and one male participant had *Mycoplasma genitalium*, but this bacterium was not detected in their spouse's sample. Similarities in the microbial diversity were rare, with the same microorganisms being highly abundant in the microbiota of one spouse (Fig. 3).

Speaking of compositional similarities between cervical and semen samples (Fig. 4), 4 (8 %) married couples had absolutely healthy microbiotas; in 23 (46 %) couples the partners shared at least 1 microorganism; in 23 couples microbiotas were absolutely different.

DISCUSSION

Real-time PCR-based assays *Androflor* and *Femoflor* provide comprehensive data about the microbiotas of sexual partners facilitating diagnosis of microbial imbalances in their semen and cervical secretions. *Androflor* can differentiate between *Ureaplasma urealyticum* and its biovar *Ureaplasma parvum*, surpassing other tests. Our study demonstrated the presence of a great variety of microorganisms in the ejaculate and cervical secretions, though the participants were relatively healthy and did not have any complaints apart from infertility. The comparison of male and female samples showed that half of the couples had compositionally different microbiotas, which confirms that the microbiota is unique in every member of the couple. Therefore, doctors should refrain from administering identical therapies to the sexual partners in the absence of data on the microbial diversity and abundance of their microbiotas.

CONCLUSIONS

Our study demonstrates that both sexual partners should be tested for infection in order to effectively diagnose genitourinary dysbiosis. This task can be successfully solved with *Androflor* and *Femoflor* reagent kits that were designed for performing qualitative and quantitative real-time PCR and can measure microbial concentrations in the sample. Based on the PCR data, the doctor can prescribe adequate treatment for each partner.

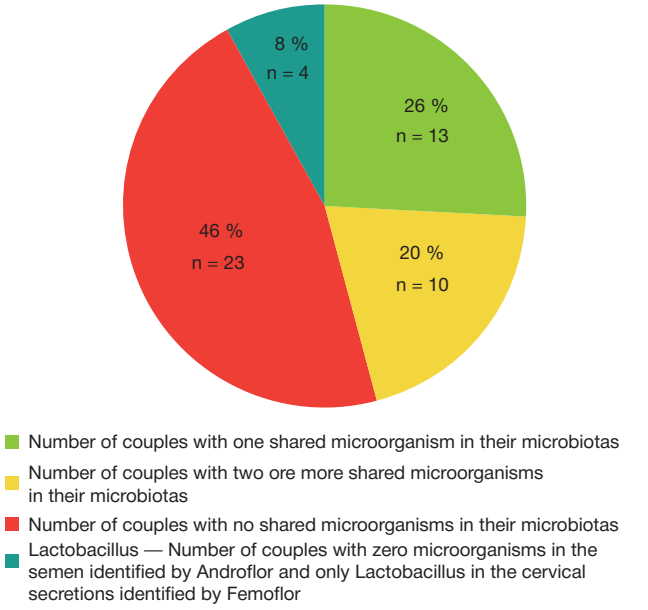

Fig. 4. Degree of similarity of semen and cervical microbiotas of 50 studied couples

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NORMAL VAGINAL MICROBIOTA: PATIENT'S SUBJECTIVE EVALUATION, PHYSICAL EXAMINATION AND LABORATORY TESTS

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Criteria of normality for the vaginal microbiota of healthy women are still a subject of discussion. A decision to assign a study participant to a group of healthy individuals is quite subjective if based on the absence of complaints and physical examination only, which renders study results ambiguous. Below we compare occurrence of the normal vaginal flora and vaginal dysbiosis in women divided into 3 groups according to the examination type (patient's subjective evaluation of her condition, physical examination, and laboratory tests). We examined 234 women of reproductive age from Yekaterinburg (mean age was 30.3 ± 6.6 years). Microbiota composition and lactobacillus diversity (*L. crispatus*, *L. iners*, *L. jensenii*, *L. gasseri*, *L. johnsonii*, *L. vaginalis*) were evaluated by real-time polymerase chain reaction using the Femoflor assay and reagent kits by DNA-Technology, Russia. One in 5 women of reproductive age who had no health complaints was found to have dysbiosis. The normal microbiota of those women was dominated mostly by *L.iners*, while dominant *L. crispatus* were observed in every third participant. Prevailing *L. crispatus* were also found in the normal microbiota of 46.2 % of women who were considered healthy based on the doctor's examination and laboratory tests. Thus, clinical evaluation of the female lower reproductive tract can be compromised by doctor's subjectivity if not supported by laboratory tests and may overlook vaginal dysbiosis in the patient.

Keywords: vaginal microbiota, vaginal lactobacilli, *Lactobacillus iners*, *Lactobacillus gasseri*, *Lactobacillus crispatus*, normal vaginal flora, dysbiosis

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НОРМАЛЬНОЕ СОСТОЯНИЕ МИКРОБИОЦЕНОЗА ВЛАГАЛИЩА: ОЦЕНКА С СУБЪЕКТИВНОЙ, ЭКСПЕРТНОЙ И ЛАБОРАТОРНОЙ ТОЧЕК ЗРЕНИЯ

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Критерии нормы для микробиоценоза влагалища здоровой женщины остаются предметом для дальнейшего изучения. Субъективный характер формирования группы «здоровых женщин» при проведении исследований не позволяет считать получаемые результаты однозначными. В данной работе мы сравнивали частоту встречаемости нормоценозов различных типов и дисбиоза влагалища у женщин, поделенных на группы в зависимости от характера их обследования (мнение женщины, осмотр врача, микроскопическое исследование). Были обследованы 234 женщины репродуктивного возраста из г. Екатеринбурга (средний возраст — 30,3 ± 6,6 лет). Оценку микробиоценоза и определение видового состава лактобацилл (*L. crispatus*, *L. iners*, *L. jensenii*, *L. gasseri*, *L. johnsonii*, *L. vaginalis*) провели методом полимеразной цепной реакции с детекцией результатов в режиме «реального времени» с использованием тест-системы «Фемофлор» («НПО ДНК-Технология», Россия) и наборов реагентов для научного применения той же компании-производителя. У каждой пятой женщины репродуктивного возраста, считающей себя здоровой, был выявлен дисбиоз. Нормоценоз в этой группе чаще всего характеризовался преобладанием *L. iners*, а вариант нормоценоза с преобладанием *L. crispatus* определяли только у каждой третьей женщины. В то же время вариант нормоценоза с преобладанием *L. crispatus* был обнаружен у 46,2 % женщин, которые были отнесены к группе клинически здоровых на основании опроса, осмотра врача и микроскопического исследования. Это говорит о том, что клиническая оценка состояния нижних отделов гениталий без учета данных микроскопии носит субъективный характер и в ряде случаев не позволяет выявить у пациентки дисбиоз влагалища.

Ключевые слова: микробиоценоз влагалища, вагинальные лактобациллы, *Lactobacillus iners*, *Lactobacillus gasseri*, *Lactobacillus crispatus*, нормоценоз, дисбиоз

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Criteria have not been ultimately defined yet for a normal vaginal microbiota of a healthy woman. A microbiota of an individual woman beneficial for her reproductive health may not be a close match to the population norm which is largely determined by a study design, since it describes the frequency of vaginal flora variations in women recruited according to certain inclusion criteria, such as ethnicity, area of residence, age, job, etc.

Extensive data have been collected under the Human Microbiome Project about the vaginal flora of women representing different ethnic groups [1–6]. The project employed whole-genome sequencing enabling identification of all microorganisms that constitute the vaginal microbiome. Some authors use a classification of vaginal microbiota types based on the proportion of dominant bacteria; if lactic bacteria are the most abundant in the community, which is an indicator of vaginal health, then microbiotas are classified according to the dominant lactobacilli species [6]. Consequently, researchers distinguish between 5 major types of microbial communities inhabiting the female reproductive tract [6]:

1. type I — normal flora dominated by *Lactobacillus crispatus*,
2. type II — normal flora dominated by *L. gasseri*,
3. type III — normal flora dominated by *L. iners*,
4. type IV — dysbiotic flora dominated by obligate anaerobes,
5. type V — normal flora dominated by *L. jensenii*.

Although all types of microbial communities listed above were identified in women of all ethnic groups, their prevalence varied depending on the ethnicity. For example, type IV (dysbiosis) was observed in 40.6 % of black and 38.1 % of Hispanic women who were shown to be at an increased risk of miscarriage associated with bacterial vaginosis (BV). At the same time, type I was more common for Caucasian participants (45.4 %). The dysbiotic microbiota was observed only in 10.3 % of white women. Correlations were established between the normal microbiota dominated by *L. crispatus* and a lower vaginal pH [6]. Strong protective effects of *L. crispatus* were associated with high colonization capacity and increased production of hydrogen peroxide and lactic acid [6–8] To our knowledge, no similar studies have been carried out in the Russian population so far.

In most cases, descriptions of the vaginal microbiome are based on the data obtained from asymptomatic women, i.e. those who consider themselves healthy. However, complaints or the lack of thereof are always subjective, because a patient does not have a reference value to compare her condition to; besides, the idea of normality varies culturally and socially. Visual assessment of the vaginal mucosa and vaginal secretions by the doctor is subjective. Therefore, criteria for normality are incomplete without lab tests. It is unclear how much the "normal" vaginal microbiota varies across studies depending on the criteria applied.

Femoflor, a real-time polymerase chain reaction-based (PCR) assay, was introduced into clinical practice in 2008. About the same time, criteria were proposed for the assessment of the statusof the vaginal flora [9]. Genotyping of vaginal lactobacilli entailed the need to revise previously used criteria for normality.

This study aimed to describe some compositional aspects of the vaginal microbiota, including the diversity of lactobacilli, in reproductive-age women from Yekaterinburg who considered themselves healthy, based on the criteria applied to the norm group.

METHODS

The study recruited 234 women aged 18 to 45 (mean age was 30.3 ± 6,6 years) who presented at the Medical Center *Harmony* (Yekaterinburg) for a gynecology check-up over the period from 2011 to 2015. All women considered themselves healthy and had no complaints indicative of vaginal inflammation. Exclusion criteria were: sexually transmitted obligate pathogens, HIV, HBV or HCV, and systemic or local antibiotic therapy in the preceding 4 weeks.

All women were examined once. Vaginal microbiota samples were analyzed using *Femoflor-16* real-time PCR-based assay (R&D DNA-Technology, Russia). Six species of *Lactobacilli* were quantified: *L. crispatus*, *L. iners*, *L. jensenii*, *L. gasseri*, *L. johnsonii*, and *L. vaginalis*, by real-time PCR, but for this purpose we used a reagent kit for scientific research by the same vendor.

Microscopy of the vaginal swab was performed using Romanovsky-Giemsa staining. The status of the vaginal microbiota was assessed based on the criteria proposed by Kira E. F. in 2001 [10].

The participants were questioned about their vaginal health. Vaginal examination with mirrors was conducted in all women to assess the status of the vaginal mucosa and quality of vaginal discharge.

Statistical analysis was performed using WinPepi. Two-tailed Fisher test was performed to estimate differences in the prevalence of different microbial communities in the participants.

RESULTS

All study participants (Main group) were divided into two subgroups. Subgroup 1 included 125 women who had no complaints and no signs of vaginal inflammation detected during visual examination by the gynecologist. Subgroup 1 consisted of 52 women from Subgroup 1 who had no complaints, no visual signs of vaginal pathology and no pathogenic shifts in the microbiota revealed by microscopy [10].

Depending on the proportion of lactobacilli that normally dominate the healthy microbiota and the proportion of opportunistic microorganisms (OMs), we identified 5 types of microbial communities:

1. normal flora, type I — proportion of *Lactobacillus spp.* > 80 %, dominated by *L. Crispatus*,
2. normal flora, type II — proportion of *Lactobacillus spp.* > 80 %, dominated by *L.gasseri prevailing*,
3. normal flora, type III — proportion of *Lactobacillus spp.* > 80 %, dominated by *L. Iners*,
4. normal flora, type IV— proportion of *Lactobacillus spp.* > 80 %, dominated by *L. jensenii/L. vaginalis*,
5. dysbiosis — proportion of *Lactobacillus spp.* < 80 %, proportion of OMs > 20 %.

Prevalence of different types of normal flora and dysbiosis is shown in Figure.

Dysbiosis was detected in 19.7 % of all examined women (Main group); the vaginal microbiota of 80 % of the participants was normal. Type III of the vaginal flora was the most common type observed in 38.9 % of the participants (Figure). The second prevailing type was type I (dominated by *L. crispatus*); it was found in 30.8% of women who considered themselves healthy.

No significant differences were revealed in the microbial composition of the vaginal microbiota between subgroup 1

(women found healthy by the visual examination) and all other participants. Dysbiosis was observed in 19.2 % of the participants in subgroup 1. Type I of the vaginal flora was detected in 31.2 % of the examined women, and type III was observed in 37.6 % of the patients. Types II and IV were rare in subgroup 1 and in all the participants in general.

Compositionally, the vaginal microbiota of subgroup 1 differed significantly from that of 2 other groups: the dysbiotic type was 5 times less common (3.8 % vs. 19.7 % in main group and 19.2 % in subgroup 1, $p < 0.01$). The normal flora dominated by *L. crispatus* was significantly more common in subgroup 1: this microbiota type was observed in almost half of the examined women. Prevalence of other microbiota types varied: type II (dominated by *L. gasseri*) and type IV (dominated by *L. jensenii/L. vaginalis*) were slightly more common than other types, while type III (dominated by *L. iners*) was less common. The difference, however, was insignificant, which may be due to the small number of women who carried these microbiota types.

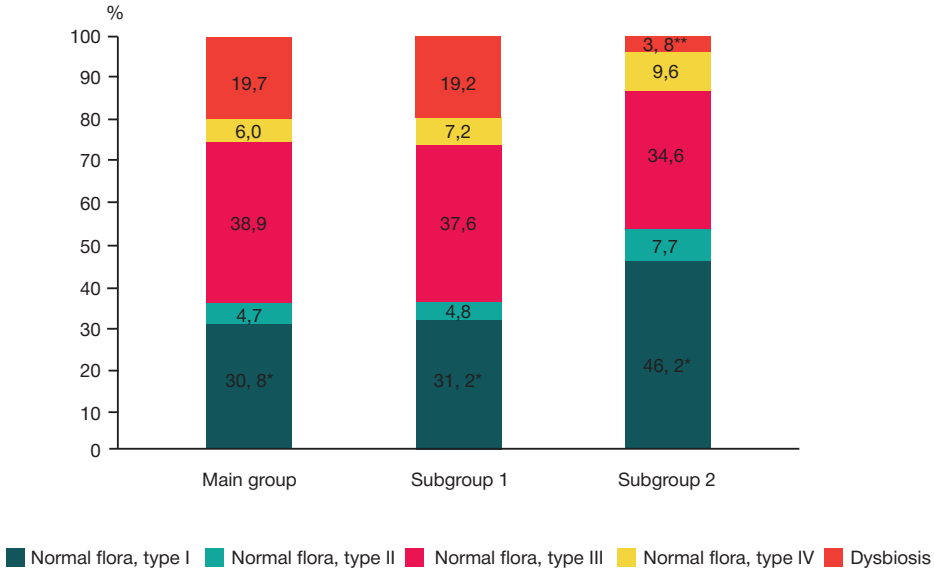
Thus, one out of 5 women who considered herself healthy (main group) was diagnosed with vaginal dysbiosis. A similar result was obtained for those women who were found healthy after the visual examination by the gynecologist (subgroup 1). In contrast, almost half of the women from subgroup 1, whose vaginal health was evaluated using microscopy, had type 1 vaginal flora — the most beneficial type of microbial community dominated by *L. Crispatus*. In this group, dysbiosis was observed in a few patients only.

DISCUSSION

The obtained data demonstrate that criteria used to form norm groups significantly affect the outcome of the study and should be carefully elaborated when working on a study design. How woman sees her health is subjective; the same is true for a visual examination performed by the doctor. Patient's and doctor's opinions alone unsupported by lab tests may result in the underdiagnosis of the dysbiotic state and untimely treatment of vaginal dysbiosis, which will affect woman's reproductive health.

In this light, it is interesting to compare our results with the data published by Ravel et al. [6]. They analyzed vaginal microbiotas in 4 ethnic groups (Caucasian, Asian, Hispanic and Afro-American). Study participants considered themselves healthy at the time of the examination, in spite of the fact that some of them had not seen a gynecologist for a check- for several years, similar to main group in our study. Results of the comparative analysis are presented in Table.

Vaginal microbiotas of women in our study differed from those of other ethnicities (the study was conducted in the USA, in Baltimore and Atlanta). In our opinion, it would be incorrect to estimate significance of differences between the two studies because methods used for the assessment of the vaginal microbiota status were different. However, in our study dysbiosis was detected 2 times more often than in the Caucasian group; its prevalence was comparable to the prevalence in the Asian group. The most beneficial microbiota type was 1.5 less



Prevalence of different types of the vaginal microbiota in women with no visual signs of vaginal inflammation (* — $p < 0.05$ and ** — $p < 0.01$ when comparing main group and subgroup 1 and subgroups 1 and 2)

Prevalence of various microbiota types in women of different ethnic groups who considered themselves healthy

Vaginal flora type	Study by Ravel et al., 2011 (whole-genome sequencing) [6]				Our data (real-time PCR)
	Caucasian (n = 98)	Asian (n = 97)	Hispanic (n = 97)	Afro-American (n = 104)	Russian, Yekaterinburg (n = 234)
Normal flora, type I	45,4 %	25,0 %	14,4 %	22,1 %	30,8 %
Normal flora, type II	8,2 %	5,2 %	7,2 %	4,8 %	4,7 %
Normal flora, type III	26,8 %	42,7 %	36,1 %	31,5 %	38,9 %
Normal flora, type IV	9,3 %	7,3 %	4,2 %	1,0 %	6,0 %
Dysbiosis	10,3 %	19,8 %	38,1 %	40,6 %	19,7 %

common in the Russian women than in the Caucasian group, but more common than in other ethnic groups. On the whole, in our study vaginal microbiota composition was similar to that of Asian women. Further research is necessary to compare the results of our study with the results of similar studies conducted in other Russian regions.

CONCLUSIONS

Vaginal dysbiosis detected by real-time PCR is common in reproductive-age women who consider themselves healthy. It was observed in one out of five patients. Normal vaginal flora was usually dominated by *L. Iners*; another type of normal flora dominated by *L. crispatus* was observed in every third woman. Normal flora dominated by *L. crispatus* was present in 46.2 % of women who were assigned to the norm group based on their own opinion, visual examination and microscopy data. Without microscopy, clinical assessment of the vaginal status is subjective and may not detect dysbiosis in a number of patients.

Variations in the vaginal microbiota composition in women from Yekaterinburg and differences from the vaginal flora of Caucasian women may be associated with ethnic diversity of female population of the Ural region.

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PREVALENCE OF *LACTOBACILLUS INERS* IN THE VAGINAL MICROBIOTA OF WOMEN WITH MODERATE DYSBIOSIS IS ASSOCIATED WITH CLINICAL SYMPTOMS OF INFECTIOUS INFLAMMATORY CONDITION OF THE VAGINA

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Moderate vaginal dysbiosis is a shift in normal vaginal microbiota composition characterized by increased levels of opportunistic microbes and an ordinary high proportion of lactobacilli that make up 20 to 80 % of the total microbial population of the vagina. Some women with vaginal dysbiosis do not show any symptoms of the infectious inflammatory condition (IIC), which raises the question of whether their dysbiosis should be corrected. We studied the association between some parameters of the microbiota and clinical symptoms of IIC in female patients with moderate vaginal dysbiosis. Participants were distributed into two groups: group 1 included patients with clinical symptoms of IIC (n = 91), group 2 was comprised of asymptomatic patients (n = 44). Mean age was 26.9 ± 6.9 years. Vaginal microbial communities were studied using real-time polymerase chain reaction assays. Levels of six Lactobacillus species were measured in the vaginal discharge: *Lactobacillus crispatus*, *L. iners*, *L. jensenii*, *L. gasseri*, *L. johnsonii*, and *L. vaginalis*. We found that *L. iners* dominated the microbiota of 45 (49.5 %) symptomatic patients and only 9 (20.5 %) asymptomatic individuals (p = 0.002), unlike *L. gasseri* that significantly prevailed in the samples of asymptomatic patients: 23 (52.3 %) women vs 21 (23.1 %) in the group of patients with clinical signs of IIC (p = 0.001).

Keywords: vaginal microbiota, vaginal lactobacilli, *Lactobacillus iners*, *Lactobacillus gasseri*, moderate vaginal dysbiosis

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ПРЕОБЛАДАНИЕ *LACTOBACILLUS INERS* В МИКРОБИОЦЕНОЗЕ ВЛАГАЛИЩА ЖЕНЩИН С УМЕРЕННЫМ ДИСБИОЗОМ АССОЦИИРОВАНО С НАЛИЧИЕМ КЛИНИЧЕСКИХ ПРИЗНАКОВ ИНФЕКЦИОННО-ВОСПАЛИТЕЛЬНОЙ ПАТОЛОГИИ ВЛАГАЛИЩА

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Умеренный дисбиоз влагалища является переходным типом вагинального микробиоценоза, для которого характерно увеличение количества и доли условно-патогенных микроорганизмов при сохранении высокой доли лактофлоры — на уровне 20–80 % от общей микробной биомассы. У части женщин данное состояние микробиоценоза не сопровождается клиническими признаками инфекционно-воспалительной патологии влагалища (ИВП), и возникает вопрос о целесообразности коррекции умеренного дисбиоза в этом случае. В исследовании оценивали взаимосвязь между отдельными микробиологическими показателями и наличием клинических проявлений ИВП у пациенток с умеренным дисбиозом вагинальной микробиоты. Были сформированы две группы участниц: группа 1 — пациентки с клиническими признаками ИВП (n = 91), группа 2 — клинически здоровые женщины (n = 44). Средний возраст женщин составил 26,9 ± 6,9 лет. Микробиоценоз исследовали методом полимеразной цепной реакции в режиме «реального времени». Провели количественную оценку 6 видов лактобацилл в вагинальном отделяемом: *Lactobacillus crispatus*, *L. iners*, *L. jensenii*, *L. gasseri*, *L. johnsonii*, *L. vaginalis*. Было установлено, что *L. iners* преобладает в микробиоценозе 45 (49,5 %) пациенток с признаками ИВП, тогда как у клинически здоровых женщин преобладание данного вида лактобацилл зафиксировали в 9 (20,5 %) случаях (p = 0,002). *L. gasseri*, наоборот, достоверно чаще преобладал в образцах, полученных от клинически здоровых пациенток: 23 (52,3 %) случая против 21 (23,1 %) в группе пациенток с клиническими признаками ИВП (p = 0,001).

Ключевые слова: микробиоценоз влагалища, вагинальные лактобациллы, *Lactobacillus iners*, *Lactobacillus gasseri*, умеренный дисбиоз влагалища

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Numerous studies show that the vaginal microbiota of healthy women is dominated by *Lactobacillus* [1–4]. Lactobacilli are thought to ensure colonization resistance of the vaginal microbial community. The vaginal epithelium can be colonized by other microbes, but they are less abundant in healthy women.

Many vaginal microorganisms are not so readily culturable or completely unculturable [5–8], including some *Lactobacillus* species that refuse to grow on standard media. It was shown that one of the most prevalent species, *Lactobacillus iners*, cannot grow on Sharpe (MRS) and Rogosa agars used to culture lactobacilli [9]. Therefore, culture-based studies provide very scarce data on the diversity of species constituting the vaginal microbial community. To date, the most comprehensive results can be achieved using methods of molecular genetics.

Health of the vaginal microbiota is determined by the abundance of lactobacilli (no less than 80 % of all species isolated from the sample) measured by real-time polymerase chain reaction assays [10]. If lactobacilli constitute 20 to 80 % of the whole microbial community, the vaginal microbiota is considered moderately dysbiotic. Moderate dysbiosis is very often asymptomatic; therefore, it presents a particular interest for researchers and health professionals and raises the question of whether it is necessary to treat this condition in the absence of signs of vaginal inflammation.

Of importance is identification of microbiological markers associated with clinical signs of vaginal inflammation in patients with moderate dysbiosis. Studies of the diversity of lactobacilli in the vaginal microbiota of reproductive-age women showed that prevalence of some lactobacilli varies in patients with different types of vaginal flora [11]. Perhaps, there is an association between the diversity of lactobacilli in patients with moderate dysbiosis and the presence of subjective symptoms and objective signs of vaginal inflammation. We cannot rule out the possibility that clinical manifestations of pathology in moderate dysbiosis depend on the diversity and abundance of opportunistic bacteria inhabiting the vagina.

The aim of this study was to estimate the correlation between some microbiological characteristics and the presence of clinical signs of vaginal infection in patients with moderate vaginal dysbiosis.

METHODS

The study was carried out in 135 women with moderate vaginal dysbiosis aged 18 to 53 (mean age was 26.9 ± 6.9 years), outpatients of the Medical Center *Harmony* (Yekaterinburg) in 2011–2016. Exclusion criteria were HIV, parenteral hepatitis, sexually transmitted infections, namely *Treponema pallidum*, *Neisseria gonorrhoeae*, *Chlamydia trachomatis*, *Mycoplasma genitalium*, and *Trichomonas vaginalis*, and antimicrobial therapy started as early as 4 weeks before the study.

Samples (posterolateral vaginal wall swabs) were collected into Eppendorf tubes containing 1 ml sodium chloride solution. DNA was extracted using the Proba-GS reagent kit (R&P DNA-Technology, Russia). Abundance and diversity of species in the samples were evaluated by real-time PCR and the Femoflor reagent kit (R&P DNA-Technology). Identification and quantification of 6 *Lactobacillus* species (*Lactobacillus crispatus*, *L. iners*, *L. jensenii*, *L. gasseri*, *L. johnsonii*, *L. vaginalis*) was done by real-time PCR with reagent kits for scientific research (R&P DNA-Technology) and the DT-96 PCR detection system by the same vendor.

Patients were questioned about their complaints and examined to identify clinical signs of infection-induced inflammation of the lower genital tract.

Statistical analysis was performed using Microsoft Office Excel 2007. Significance of differences was estimated by the two-tailed Fisher's test using WinPepi software.

The study was approved by the Ethics Committee of the Ural State Medical University (Protocol No. 4 dated May 05, 2015). All patients gave their informed consent.

RESULTS

All patients were divided into two groups depending on the presence of clinical signs of an inflammatory infection (II) in the lower genital tract. Group 1 consisted of 91 patients with clinical signs of II, group 2 included 44 healthy women. We attempted to establish associations between the proportion of lactobacilli in the microbiota, the dominant species of lactobacilli, the dominant species of opportunistic microorganisms (OMs), and II.

Based on the proportion of lactobacilli (20–40 %, 40–60 % and 60–80 %), all patients were divided into 3 subgroups. Then the relative share of each subgroup in groups 1 and 2 was estimated (Fig. 1). The difference between the groups was insignificant.

Prevalence of dominant *Lactobacillus* species in groups 1 and 2 was different (Fig. 2). *L. iners* was significantly more common in group 1 (patients with II) than in group 2: 45 women (49.5 %) vs. 9 (20.5 %), respectively (p = 0.002). *L. gasseri*, on the contrary, was significantly more common in group 2

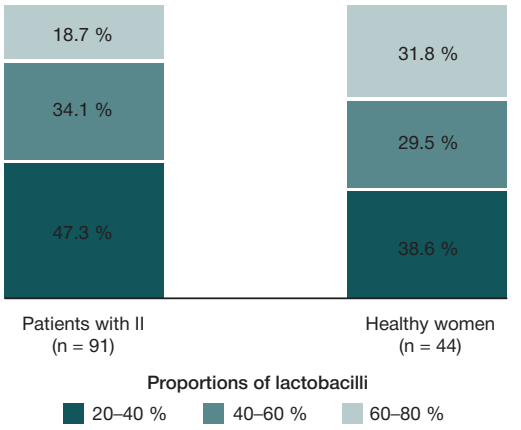

Fig. 1. Proportions of lactobacilli in the vaginal microbiota of women with moderate dysbiosis in the presence and absence of clinical signs of inflammatory infection (n = 135)

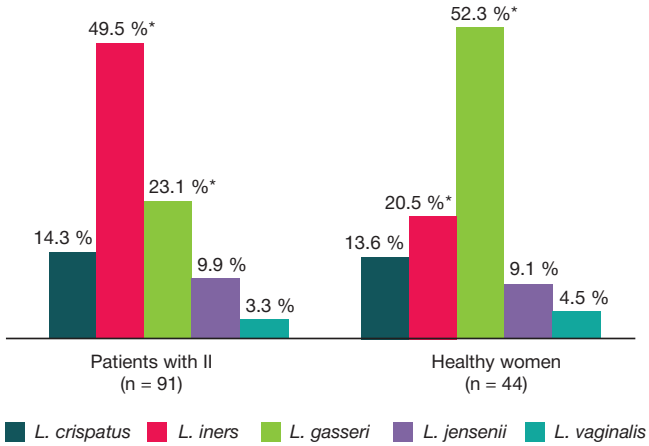

Fig. 2. Prevalence of lactobacilli species in the vaginal microbiota of women with moderate dysbiosis in the presence and absence of clinical signs of inflammatory infection (n = 135). Asterisks represent significantly different values (p < 0.01)

Prevalence of opportunistic pathogens in the vaginal microbiota of women with moderate vaginal dysbiosis in the presence or absence of clinical signs of inflammatory infection (n = 135)

Dominant opportunistic pathogen	Patients with II (n = 91)	Healthy women (n = 44)
Enterobacteriaceae family	3 (3.3 %)	0
Streptococcus spp.	6 (6.6 %)	6 (13.6 %)
Enterococcus spp.	0	2 (4.5 %)
Gardnerella vaginalis/Prevotella bivia/Porphyromonas spp.	50 (54.9 %)	24 (54.5 %)
Eubacterium spp.	8 (8.8 %)	5 (11.4 %)
Sneathia spp./Leptotrichia spp./ Fusobacterium spp.	2 (2.2 %)	0
Megasphaera spp./Veilonella spp. /Dialister spp.	4 (4.4 %)	3 (6.8 %)
Clostridium spp./Lachnobacterium spp.	3 (3.3 %)	0
Peptostreptococcus spp.	0	1 (2.3 %)
Atopobium vaginae	15 (16.5 %)	3 (6.8 %)

(healthy women) than in group 1: 23 patients (52.3 %) vs. 21 (23.1 %), respectively (p = 0.001). Prevalence of *L. crispatus*, *L. jensenii* and *L. vaginalis* in both groups was comparable.

Opportunistic pathogens were represented by dominant *Gardnerella vaginalis/ Prevotella bivia/Porphyromonas spp*. (GPP) in every second woman with or without clinical signs of II. Other OMs were far less common. No significant difference was revealed between OM prevalence in groups 1 and 2 (see the Table).

DISCUSSION

Study results demonstrate that lactobacilli inhabiting the vagina of reproductive-age women are represented mainly by *L. crispatus*, *L. iners*, *L. gasseri*, and *L. jensenii*, which is consistent with the results of other studies [1, 2, 12, 13]. It is noteworthy that *L. iners* and *L. gasseri* dominate the *Lactobacillus* community in patients with moderate dysbiosis. A number of researchers have demonstrated that the presence of these lactobacilli is associated with an increased risk of bacterial vaginosis and poor pregnancy outcome [14–16]. Previously, we showed that *L. gasseri* can dominate the vaginal microbiota of patients with moderate dysbiosis [11]. Frequent detection of *L. gasseri* as a dominant species in patients without clinical signs of II, whose microbiota can be described as moderately dysbiotic, prompts us to assume that it can be a normal variant of the healthy vaginal flora and does not require any treatment. At the same time, moderate dysbiosis characterized by dominant *L. iners* is very often accompanied by clinical signs of II. Moreover, *L. iners* dominance is associated with an increased risk of marked vaginal dysbiosis [11]. Recent studies show that *L. iners* are higly adaptable and can survive in the presence of abundant Oms [17, 18]. Therefore, dominance of *L. iners* is a very unfavorable factor and requires medical correction.

We were unable to identify an association between the proportion of lactobacilli in the microbiota and the presence of II in patients with moderate vaginal dysbiosis. However, the obtained results may have been influenced by a small patient sample size, which means that such an association remains a possibility.

In more than half of patients with moderate vaginal dysbiosis, opportunistic bacteria were represented by GPP. In the studies *in vitro Gardnerella vaginalis*, a member of the GPP group, was shown to have a high adhesion capacity [19, 20] and stimulate growth of other OMs, including *Prevotella bivia*, also a GPP representative [19]. It was hypothesized that *G.vaginalis* could be the first microorganism that colonizes the vagina and prepares the environment for other pathogens [21, 22]. This can explain high prevalence of GPP as dominant opportunistic pathogens in patients with moderate dysbiosis. It is possible that as dysbiosis progresses, the contribution of other OMs to pathology increases; this may be true for *Atopobium vaginae*, a microorganism associated with vaginal dysbiosis [23–26]. In our study *A. vaginae* was twice more common in patients with II than in healthy women. However, the difference was not statistically significant. We assume that the lack of significance was due to the small number of healthy women in group 2.

CONCLUSIONS

Dominance of *Lactobacillus iners* in the *Lactobacillus* community of the vaginal microbiota of women with moderate dysbiosis is associated with clinical signs of the infection of the lower genital tract, while dominance of *L. gasseri* is typical for clinically healthy women with moderate dysbiosis. Thus, these microorganisms can be used as microbiological markers when it is unclear whether dysbiosis requires treatment.

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EFFECTIVENESS AND SAFETY OF TREATMENT WITH DOMESTIC CEPEGINTERFERON ALPHA-2B IN PATIENTS WITH CHRONIC HEPATITIS C INFECTION. ACTUAL CLINICAL EXPERIENCE

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Interferon-based regimens for chronic hepatitis C (HCV) are quite common, just like interferon-free treatments, and are extensively used in Russia because interferon is widely available to most patients. In 2013 the original Russian drug cepeginterferon alpha-2b (cepegIFN alpha-2b marketed as Algeron by Biocad, Russia) was introduced into clinical practice. The aim of this study was to assess effectiveness and safety of cepegIFN alpha-2b as part of the combination therapy with ribavirin in patients with chronic HCV infection. The study was conducted over the period from 2014 to 2016 and recruited 37 patients with chronic genotype 1 HCV infection: 22 men and 15 women (mean age of 42.0 ± 5.2 years). All of them received the following combination antiviral therapy (AT): 1.5 μg/kg cepegIFN alpha-2b once a week and 15 μg/kg ribavirin daily over the period of 48 weeks. Effectiveness of AT was assessed by the rate of sustained virological response (SVR), i. e. aviremia achieved 24 weeks after the onset of treatment. In our SVR was observed in 26 patients (70.3 %). Adverse effects seen in the course of AT were typical of interferon-based drugs and ribavirin. CepegIFN alpha-2b dosage was corrected in two patients who developed neutropenia; ribavirin dosage was corrected in 3 patients who developed anemia. Based on the obtained results, we recommend including cepegIFN alpha-2b into the combination antiviral therapy in patients with chronic HCV infection.

Keywords: chronic hepatitis C, HCV infection, antiviral therapy, cepeginterferon alpha-2b, ribavirin, virological response, treatment effectiveness

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ЭФФЕКТИВНОСТЬ И БЕЗОПАСНОСТЬ ПРИМЕНЕНИЯ ОТЕЧЕСТВЕННОГО ЦЕПЭГИНТЕРФЕРОНА АЛЬФА-2В В ТЕРАПИИ ХРОНИЧЕСКОГО ГЕПАТИТА С. ОПЫТ РЕАЛЬНОЙ КЛИНИЧЕСКОЙ ПРАКТИКИ

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В настоящее время для лечения хронического гепатита С (ХГС) используют как безинтерфероновые, так и интерферонсодержащие схемы противовирусной терапии. Последние достаточно широко используются в России за счет доступности препаратов интерферона широким слоям населения. С 2013 г. в клинической практике используется оригинальный российский препарат — цепэгинтерферон альфа-2b (цепэгИФН альфа-2b; торговая марка «Альгерон», «Биокад», Россия). Целью настоящего исследования являлась оценка эффективности и безопасности цепэгИФН альфа-2b при его применении с рибавирином для лечения пациентов с ХГС. Исследование было проведено в 2014–2016 гг., в нем приняли участие 37 пациентов с ХГС (генотип вируса 1): 22 мужчины и 15 женщин (средний возраст — 42,0 ± 5,2 года). Все они впервые получали комбинированную противовирусную терапию (ПВТ): цепэгИФН альфа-2b в дозе 1,5 мкг/кг/нед. и рибавирин в дозе 15 мг/кг/сут. в течение 48 недель. Эффективность ПВТ оценивали по частоте достижения устойчивого вирусологического ответа (УВО) — авиремии через 24 недели после ПВТ. В нашем исследовании УВО достигли 26 пациентов (70,3 %). Зарегистрированные на фоне ПВТ нежелательные явления были характерными для интерферона и рибавирина. Дозу цепэгИФН альфа-2b в связи с развитием нейтропении корректировали 2 пациентам, дозу рибавирина в связи с развитием анемии — 3 пациентам. Полученные результаты позволяют рекомендовать цепэгИФН альфа-2b для включения в схемы комбинированной противовирусной терапии для лечения больных с ХГС.

Ключевые слова: хронический гепатит С, вирус гепатита С, противовирусная терапия, цепэгинтерферон альфа-2b, рибавирин, вирусологический ответ, эффективность лечения

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Currently, 2 to 3 % of the world population are infected with hepatitis C [1, 2]. It is known that HCV infection can progress asymptomatically. Without treatment some patients develop liver fibrosis, cirrhosis, liver failure, and hepatocellular carcinoma [1–3].

Antivirals are a standard treatment for chronic hepatitis C (CHC). According to European and Russian clinical guidelines, antiviral therapy (AVT) should be prescribed to all HCV-infected patients; however, there are patients who require immediate AVT and those whose AVT can be delayed [1, 2].

There are interferon-based and interferon-free regimens for AVT. First-line therapy is based on the use of pegylated interferon alpha (PEG-IFN) in combination with ribavirin [1–3]. PEG-IFN is obtained by attaching an interferon molecule to a polymer called polyethylene glycol. The therapeutic effect of PEG-IFN is determined by interferon that exhibits antiviral, immunomodulatory and antiproliferative properties. Conjugation with polyethylene glycol increases the molecular weight of the resulting molecule, which prolongs IFN-alpha circulation in the blood.

Cepeginterferon alpha-2b, an original drug marketed as Algeron (Biocad, Russia), is one of the relatively inexpensive PEG-IFNs available on the Russian market, with polyethylene glycol molecular weight of 20kDa. It has been used in the clinical routine since 2013 [4, 5]. Unlike other interferon-based drugs, such as PEG-IFN alpha-2a or PEG-IFN alpha-2b, cepeginterferon alpha-2b has a single isomer, which makes its composition homogenious and ensures stable antiviral activity. So far, clinical trials have shown its sufficient effectiveness and an acceptable safety profile in comparison with other PEG-IFNs, and the drug was subsequently included into double and triple antiviral therapy regimens [6, 7]. It should be noted that AVT regimens based on cepegIFN-alpha 2b are available to the majority of HCV-infected Russian, due in no small part to its relatively low cost. In this light, its effectiveness, safety and tolerance should be widely discussed [5, 7].

The aim of this study was to assess effectiveness and safety of combination therapy with cepegIFN alpha-2b and ribavirin in patients with chronic HCV infection in the clinical setting.

METHODS

The study was conducted at the facilities of Agafonov Republican Clinical Hospital of Infectious Diseases, Kazan, in 2014–2016. The study enrolled 37 patients with CHC: 22 men and 15 women aged 23 to 65 years (mean age was 42.0 ± 5.2 years). Forty-five percent of patients had been infected for up to 5 years by the time of the study. All participants received a previously untried combination AVT with 1.5 μg/kg cepegIFN alpha-2b per week and 15 μg/kg ribavirin per day for 48 weeks.

In preparation for the treatment , at weeks 4, 12, 24 and 48 of the treatment, and 24 weeks after the treatment, a number of tests were carried out, including (1) the enzyme linked immunosorbent assay (ELISA) to detect the presence of anti-HCV and anti-HBsAg antibodies using the Multiskan Ascent plate reader (Agiletn Technologies, USA) and the Best reagent kit by Vector-Best, Russia; (2) determination of the viral load and genotyping of viral RNA using real-time polymerase chain reaction (sensitivity of 15 mU/ml); a viral load of $> 8 \times 10^5$ mU/ml viral RNA copies was considered high; these tests were run using the Rotor Gene-Q real-time PCR cycler (Qiagen, Germany); (3) the analysis of *IL28B* single nucleotide polymorphisms *rs8099917* and *rs12979860* (reagents used: the AmpliSense reagent kit by Interlabservice, Russia); (4) ultrasound imaging of the hepatobiliary system; (5) transient elastography of the liver with FibroScan 502 Touch (Echosens, France); (6) liver function tests, protein profile, full blood count, urinalysis; (7) ultrasound imaging of the thyroid and measuring the levels of the thyroid stimulating hormone, triiodothyronine (T3), thyroxine (T4), and thyroid peroxidase antibodies; (8) determination of ANA, AMA, ASMA, and LKM antibodies in the blood, if clinically indicated; (9) the examination of the periodontium followed by treatment, if necessary.

To assess treatment safety, we recorded every change in patients' general condition and deviations from the norm detected by blood tests. Side effects were evaluated using the CTCAE (Common Terminology Criteria for Adverse Events) [6].

Treatment effectiveness was assessed based on the frequency of sustained virologic response (SVR), or aviremia, 24 weeks after the treatment was complete.

Statistical analysis was performed using MS Excel-2007 and the Student's t-test.

The study was approved by the Ethics Committee of Agafonov Republican Clinical Hospital of Infectious Diseases (Protocol No. 4 dated December 17, 2013). All patients gave their informed consent.

RESULTS

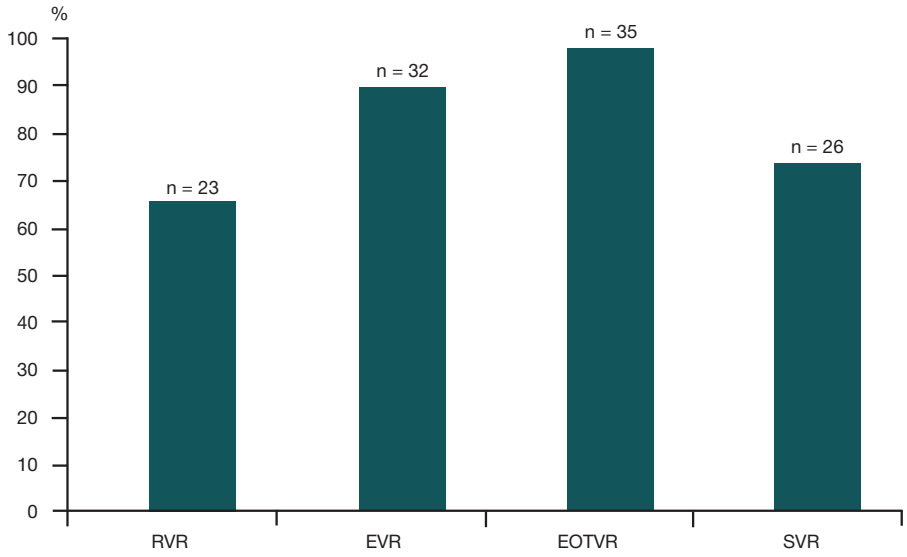
Genotyping revealed that all patients were infected with genotype 1 HCV (subtype 1b was identified in 36 patients; one patient was infected with subtype 1a). The viral load was low in 56.8 % of patients (n = 21). The samples of 84 % (n = 31) of patients were screened for *IL28B* polymorphisms to reveal that 58 % of them had favorable *IL28B* genotypes: CC (*rs12979860*) and TT (*rs8099917*).

Fibroscans detected no signs of fibrosis (F0 METAVIR) in 48.6 % of patients; mild and moderate fibrosis (F1–F2 METAVIR) was observed in 17.1 % and 14.3 % of patients, respectively. Marked fibrosis (F3–F4) was detected in 20 % of patients. There were no patients with cirrhosis.

The patients were distributed into groups based on the type of the virologic response achieved at different stages of AVT and at week 24 of posttreatment follow-up, as shown in the figure below. Rapid virologic response (RVR) at week 4 of the treatment was observed in 62.2 % of patients; early virologic response (EVR) at week 12 of the treatment was observed in 86.5 % of patients; end-of-treatment virologic response (EOTVR) was observed in 94.6% of the participants. Sustained virologic response was achieved in 26 patients (70.3 %).

Before the treatment, the majority of patients had elevated levels of alanine aminotranspherase (ALT), the average ALT level being 76.22 ± 4.77 U/l. In the course of treatment, the ALT dynamics was positive: at weeks 4 and 12 of treatment, ALT levels were down to 38.2 ± 3.11 U/l ($p < 0.05$) and 31.37 ± 1.27 U/l ($p < 0.05$), respectively. By the end of treatment, ALT was within a range of reference values in 92 % of patients (n = 34).

Treatment safety was assessed based on changes in patients' general health and deviations from the norm detected by blood tests. The observed adverse effects were typical of interferon and ribavirin: flu-like and asthenic-vegetative syndromes (weakness, poor performance at work, fatigue), and were observed in 36 patients. The flu-like syndrome occurred in the beginning of treatment and was eliminated by the intake of nonsteroidal antiinflammatory drugs. Skin reactions, such as dryness, itching, or rashes, were seen in 66.7 %. Increased



RVR — rapid virologic response at week 4 of treatment
EOTVR — end-of-treatment response at week 48 of treatment
EVR — early virologic response at week 12 of treatment
SVR — sustained virologic response at week 24 of posttreatment follow-up

Distribution of patients into groups based on the type of virologic response. Each patient can be included into more than one group depending on his/her response to therapy

irritability and mood changes were observed in 61.2 % of patients.

Blood tests indicated neutropenia, anemia and thrombocytopenia (see the Table). Grades 1 and 2 anemia (according to the CTCAE scale) was observed in 89.2 % patients. One patient was prescribed leucostim (300 μg, taken twice over the period of two weeks with a 7-day interval) at week 4 of the treatment because of neutropenia (neutrophil count went back to normal after leucostim therapy). Grades 1 and 2 thrombocytopenia was detected in 35.1 % of patients.

The dose of cepegIFN alpha-2b was corrected in 2 patients who developed neutropenia; ribavirin dose was corrected in 3 patients who developed anemia.

DISCUSSION

The obtained results demonstrate high effectiveness (70.3 % of patients with sustained virologic response) and safety of a combination AVT with cepegIFN alpha-2b in patients with chronic HCV. Our data are consistent with the results of other studies [6–11]. For example, in a randomized comparative clinical study aimed to assess effectiveness and safety of 1.5 μg/kg and 2.0 μg/kg doses of cepegIFN alpha-2b taken in combination with ribavirin [6], sustained virologic response was achieved in 71.4 % of patients (cepegIFN alpha-2b dose of 1.5 μg/kg). In another study [7], SVR was achieved in 64.6 % of patients with genotype 1 HCV, while in patients with positive predictors of treatment outcome, this proportion was 75.0 %.

No serious adverse events were observed in any of the participants; all side effects were expected and typical of pegIFN alpha and ribavirin. Therefore, a combination therapy with cepegIFN alpha-2b and ribavirin is a reasonable choice for patients with predictors of positive response to AVT (mild fibrosis, low viral load, favorable *IL28B* polymorphisms, no comorbidities).

CONCLUSIONS

Sustained virologic response to a combination therapy with a cepegIFN alpha-2b and ribavirin was achieved in 70.3 % of patients with chronic HCV infection. All adverse effects were not unexpected and did not require termination of treatment. Considering that interferon-free regimens for antiviral therapy are unavailable to the majority of the Russian population, interferon-based treatment is a good choice for patients with genotype 1 HCV due to its relatively high effectiveness and safety.

Blood counts of patients with chronic HCV infection before, during and 24 weeks after the antiviral therapy. Data are represented as M ± m, n = 37

Parameters	Observation period					
	before treatment	week 4 of treatment	week 12 of treatment	week 24 of treatment	week 48 of treatment	24 weeks after treatment
WBC × 10^9 cells/L	5.5 ± 0.16	3.4 ± 0.12**	3.3 ± 0.11**	2.7 ± 0.16***	2.8 ± 0.18***	4.2 ± 0.26
RBC × 10^{12} cells/L	4.7 ± 0.06	4.0 ± 0.08	3.6 ± 0.06*	3.4 ± 0.09*	3.5 ± 0.08*	4.4 ± 0.06
HGB, g/L	141.5 ± 2.0	116.0 ± 3.0*	113.0 ± 2.9*	111.0 ± 2.8**	112.0 ± 4.02**	137.0 ± 1.2
PLT × 10^9 cells/L	220.0 ± 8.2	161.1 ± 8.4*	157.0 ± 9.1***	171.5 ± 8.0*	151.0 ± 9.96**	224.0 ± 8.1

Note. * — $p < 0.05$, ** — $p < 0.01$, *** — $p < 0.001$ when comparing blood counts before and during the treatment.

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NG:YAG LASER HYALOIDOTOMY FOR PREMACULAR HEMORRHAGE IN BOTH EYES IN A PATIENT WITH ACUTE MYELOBLASTIC LEUKEMIA

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Premacular hemorrhage occurs in various disorders and causes sudden unilateral or bilateral visual impairment. One of the well-established techniques to treat this condition is Ng:YAG laser hyaloidotomy. Below we report a case of premacular hemorrhage in the right and left eyes of a 23-year old patient with acute myeloblastic leukemia. Ng:YAG laser hyaloidotomy was successfully performed on both patient's eyes at different puncture sites.

Keywords: premacular hemorrhage, YAG laser, posterior hyaloid membrane, acute myeloblastic leukemia

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ИАГ-ЛАЗЕРНАЯ ПУНКЦИЯ ПРИ ПРЕМАКУЛЯРНОМ КРОВОИЗЛИЯНИИ НА ОБОИХ ГЛАЗАХ У ПАЦИЕНТА С ОСТРЫМ МИЕЛОБЛАСТНЫМ ЛЕЙКОЗОМ

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Премакулярные кровоизлияния могут возникать по различным причинам и вызывают внезапное одно- или двустороннее снижение зрения. Одним из признанных вариантов лечения является ИАГ-лазерная пункция задней гиалоидной мембраны в зоне кровоизлияния. Авторы представляют случай успешной ИАГ-лазерной пункции премакулярных кровоизлияний на обоих глазах с различной локализацией точки пунктирования у 23-летнего пациента с острым миелобластным лейкозом.

Ключевые слова: премакулярное кровоизлияние, ИАГ-лазер, задняя гиалоидная мембрана, острый миелобластный лейкоз

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Premacular hemorrhages (PMHs) lead to sudden and profound vision loss. They are caused by retinal vascular disorders (diabetic retinopathy or vein occlusion), age-related macular degeneration, Valsalva retinopathy, macroaneurysms, etc. [1–6]. Up to 49 % of patients with leukemia suffer from hemorrhages in the ocular fundus, of them 6 % have PMHs [7]. In most cases these lesions resolve spontaneously within a few weeks or months, depending on the size. But for socially active patients or those with hemorrhages in both eyes or in their only healthy eye recovery should be expedited. Besides, vision restoration has a positive psychological impact on patients with severe comorbidities. Of all known treatment approaches, including pneumatic displacement therapy with tissue plasminogen activator [8, 9], vitrectomy [10], and Ng:YAG laser hyaloidotomy [11–14], the latter is the safest and the most accessible.

Below we describe a case of a 23-year old patient with acute myeloblastic leukemia with PMHs in both eyes resulting in profound visual impairment. Ng:YAG laser posterior hyaloidotomy was performed on both eyes of the patient, with a few minute break between the procedures. This case presents a particular interest, because due to the location of the hemorrhage inferior margin in the fovea of the patient's left eye, it was decided to make the opening above the fovea to avoid retinal damage. For the right eye the puncture site was conventionally chosen at the inferior margin of the lesion. Post-treatment follow-up revealed that hemorrhages resorbed differently in the right and left eyes.

Case description

A 23-year old patient with acute myeloblastic leukemia undergoing polychemotherapy presented with profound vision loss following premacular hemorrhages in both eyes six weeks earlier. Upon admission best corrected visual acuity (Vis) was 0.1 for the right eye (OD) and 0.05 for the left eye (OS); intraocular pressure (IOP) was as follows: IOP OD = 20 mmHg, IOP OS = 20 mmHg. In both eyes preretinal hemorrhages were observed in the posterior pole of the ocular fundus, extending to the fovea. The size of the lesion area in the macula was about 5 diameters of the optic disc in the right eye and 3 diameters of the disc in the left eye. We performed Ng:YAG

laser posterior hyaloidotomy above the lesion area in both eyes using the VISULAS YAG III platform (Carl Zeiss Meditec AG, Germany) with a wavelength of 1064 nm and spot diameter of 10 μm in two single bursts at E = 2 mJ in each eye with a few minute break between the procedures. The central fovea lies 500 μm below the horizontal line bisecting the optic disc [15]. In our experience, the opening in the hyaloid membrane should be made no closer than 1000 μm to the central fovea to avoid damage to the latter. Therefore, it was decided to perform hyaloidotomy of the right eye at the inferior margin of the lesion, 2500 μm below the horizontal line (Fig. 1, A). But as the inferior margin of the hemorrhage in the left eye was in the foveal area, we decided to make an opening 1000 μm above the fovea, or 500 μm above the horizontal line (Fig. 1, B).

A week after hyaloidotomy best corrected Vis OD was 0.7, best corrected Vis OS was 0.08, IOP OD was 19 mmHg, IOP OS was 19 mmHg. Areas of residual hemorrhage were observed in the macula of both eyes (Fig. 2, A). Optical coherence tomography (OCT) performed with the Avanti RTVue 100 scanner (Optovue, USA) revealed highly reflective opacities in the vitreous and an opening in the partially detached posterior hyaloid membrane in the right eye AND highly reflective opacities above the fovea (areas of residual hemorrhage) and an opening in the partially detached posterior hyaloid membrane in the left eye (Fig. 3, A). Results 5 weeks after hyaloidotomy: best corrected Vis OD = 0.7, best corrected Vis OS = 0.1, IOP OD = 18 mmHg, IOP OS = 16 mmHg; complete hemorrhage diffusion in the right eye's macula, small areas of residual hemorrhage in the left eye's fovea (Fig. 2, B). Results 14 weeks after hyaloidotomy: corrected Vis OD = 1.0, corrected Vis OS = 0.6, IOP OD = 18 mmHg, IOP OS = 16 mmHg, complete resorption of the hemorrhage in the right eye's macula, small areas of residual hemorrhage in the left eye's macula (Fig. 2, C). OCT revealed almost complete resorption of highly reflective opacities above the fovea in both eyes (Fig. 3, B). No changes in visual acuity were seen in further follow-up examinations.

Case discussion

Ng:YAG laser posterior hyaloidotomy is an effective and safe technique for treating premacular hemorrhages. Traditionally, it is performed at the inferior margin of the lesion for rapid blood drainage, which is assisted by gravity and oscillations of the detached posterior hyaloid membrane above the lesion that are in turn triggered by the oscillations of the vitreous accompanying normal eye movements. The opening must be far enough from the fovea to avoid damage to the latter. In the described clinical case indications for hyaloidotomy of the right eye were unquestionable. It was risky, however, to perform the procedure at the inferior margin of the lesion in the left eye. A decision was made to make an opening above the fovea. Obviously, considering such location of the opening, gravity will not facilitate blood drainage from the lesion area below the puncture site. But we assumed that blood drainage could be assisted by small oscillations of the detached posterior hyaloid membrane above the lesion that are a result of oscillations of the vitreous that accompany eye movements. The follow-up examinations confirmed our assumptions. But as we had expected, resorption of the hemorrhage in the left eye took longer (1 month longer) than in the right eye, in spite of a smaller lesion size.

CONCLUSIONS

Ng:YAG laser posterior hyaloidotomy performed above the lesion area is an effective and safe technique for treating premacular hemorrhages in patients with acute myeloblastic leukemia undergoing chemotherapy.

The case presented above demonstrates the possibility of treating premacular hemorrhages extending to the fovea with Ng:YAG laser puncture. The opening should be made above the fovea, unlike standard openings usually made at the inferior margin of the lesion. However, such location of the puncture site will result in slower hemorrhage resorption.

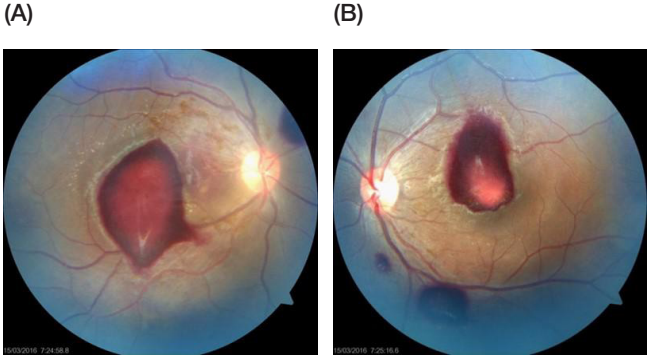

Fig. 1. Fundus photos of the right **(A)** and left **(B)** eyes of the patient upon admission to the clinic

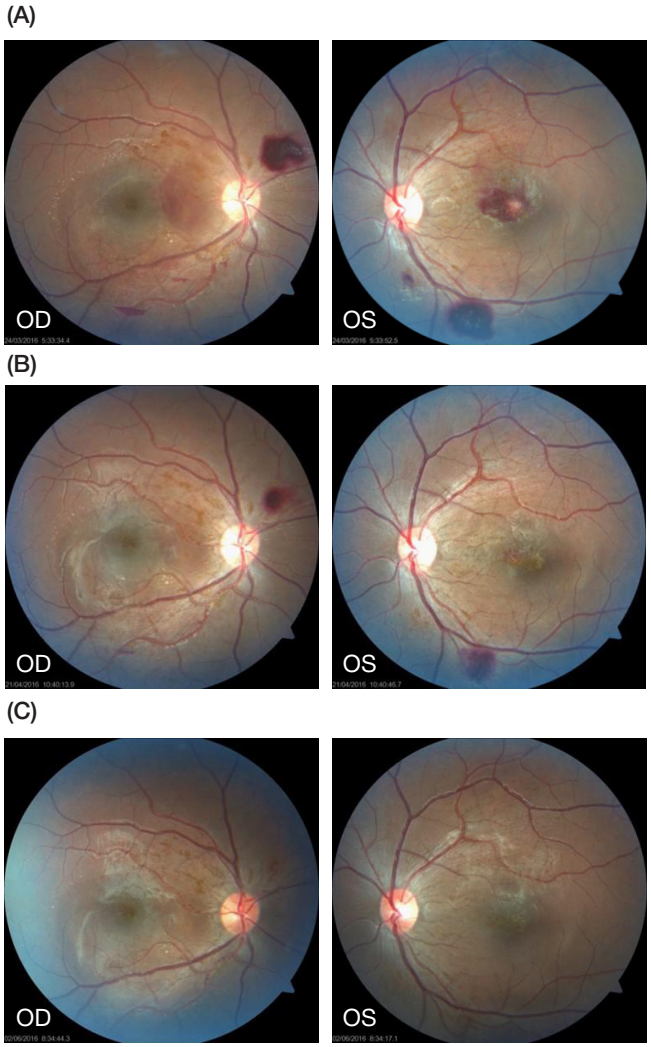

Fig. 2. Fundus photos of the right (OD) and left (OS) eyes of the patient **(A)** 1 week, **(B)** 5 weeks and **(C)** 14 weeks after Ng:YAG laser posterior hyaloidotomy

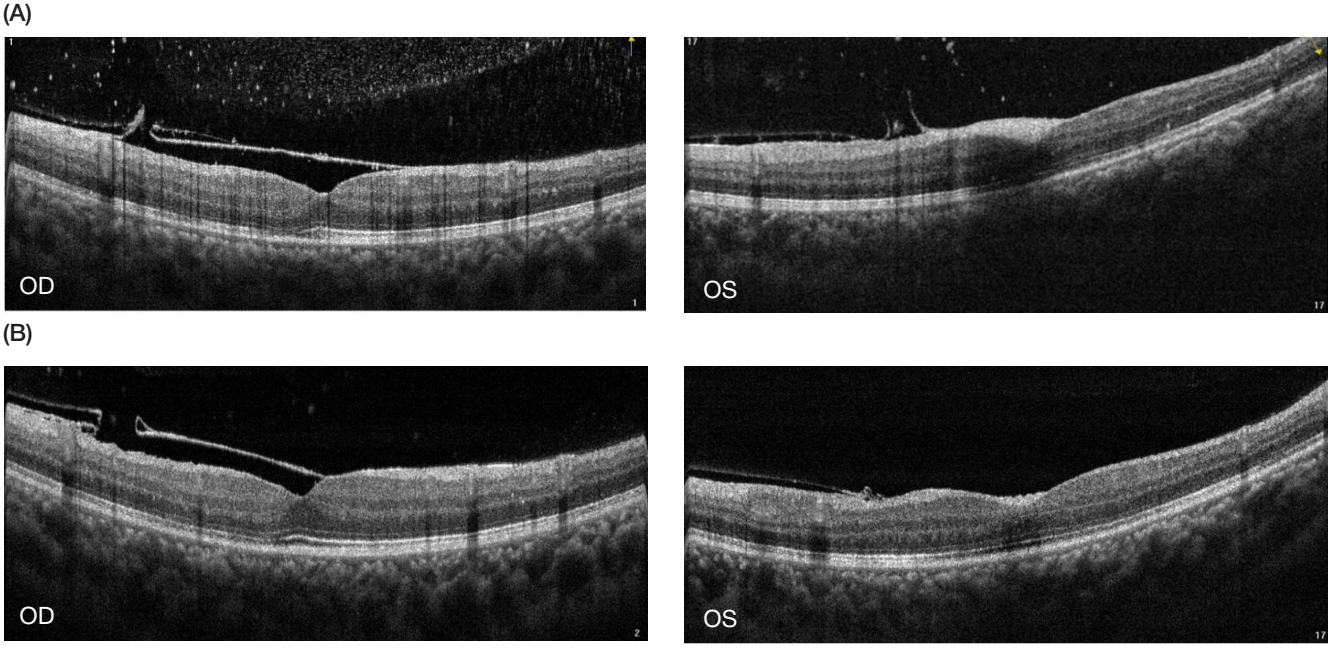

Fig. 3. Optical coherence tomography of the macula of the right (OD) and left (OS) eyes of the patient **(A)** 1 week and **(B)** 14 weeks after Ng:YAG laser hyaloidotomy

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BIOLUMINESCENCE: IS IT POSSIBLE FOR A PLANT?

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An extensive collection of plants gathered in the European part of Russia was screened for a substrate of fungal luciferase. This work was inspired by the recently discovered mechanism of bioluminescence in higher fungi and the structural similarity of fungal luciferin with some plant metabolites. Of all studied leaf extracts obtained from 200 different plants, bioluminescent activity was discovered in 10 species. Each of these species contained a plurality of active compounds. All the luminescent substrates were not identical to fungal luciferin (3-hydroxyhispidin) and were chemically unstable, rendering the attempt to isolate individual compounds for further structural characterization yet unsuccessful. This study is the first step towards engineering a self-luminescent plant based on a fungal enzyme-substrate bioluminescent system.

Keywords: fungal bioluminescence, engineered luminescent plants, luciferins, plant methabolites

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ВОЗМОЖНА ЛИ БИОЛЮМИНЕСЦЕНЦИЯ У РАСТЕНИЙ?

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На основе открытого недавно механизма биолюминесценции высших грибов и сходства структуры люциферина грибов и некоторых метаболитов растений поставлена задача поиска растений, содержащих субстрат(ы) реакции грибной люминесценции. В результате скрининга коллекции растений европейской части России обнаружено 10 видов, экстракты листьев которых проявляют биолюминесцентную активность. Установлено, что изученные виды растений синтезируют не одно, а множество активных соединений. Все люминесцентные субстраты, содержащиеся в растениях, не идентичны грибному люциферину (3-гидроксигиспидину) и химически нестабильны, что препятствует выделению индивидуальных соединений. Данное исследование можно считать первым шагом в создании автономно люминесцентного растения на базе фермент-субстратной системы высших грибов.

Ключевые слова: биолюминесценция грибов, люминесцентные биоинженерные растения, люциферины, метаболиты растений

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Bioluminescence (BL) is the emission of visible light by living organisms. The phenomenon was demonstrated *in vitro* more than a century ago by Raphaël Dubois [1]. He mixed “cold” and “hot” extracts obtained from the light organs of the beetle *Pyrophorus noctilucus*. The extract prepared with cold water comprised a heat-labile enzyme luciferase and the hot water extract contained a heat-stable luciferin. Therefore, light emission from the mixture of the two extracts was the result of a substrate-enzyme reaction. Bioluminescence as a term was first used by Harvey [2]. In all known BL systems oxygen is required for the reaction producing the oxidized luciferin. Relaxation of oxyluciferin from the excited to ground state is accompanied by light emission.

BL is widely spread among animal and fungi kingdoms, but not a one luminescent plant is known in nature [3]. The first attempt for a glowing plant engineering was undertaken more

than 30 years ago [4]. Based on the well-studied BL system of the firefly, the bioengineered plant *Nicotiana tabacum* was obtained by inserting the luciferase gene. When mixing an extract of the plant with a solution of luciferin and ATP, or when immersing the intact plant in the same solution, BL light was detected. An image of the luminous plant was demonstrated by the exposure on an X-ray film. Later some more attempts were performed [5, 6] and even a project on the creation of a luminous plant was announced [7], but no fundamentally new achievements were obtained. Our recent investigation on BL of higher fungi allowed us to believe in the possibility of more successful results using the features of this enzyme-substrate system.

BL systems are very specific within animal taxa whereas higher fungi share a uniform BL mechanism [8]. Fungal BL was considered as a two-step process first in 1961 [9]. A luciferin precursor is reduced by an NAD(P)H-dependent enzyme to a true luciferin and then luciferin is oxidized by air under luciferase catalysis to produce visible light at 520–530 nm [10]. After multiple unsuccessful attempts [11, 12] luciferin precursor was isolated from the fruiting bodies of nonluminous fungus *Pholiota squarrosa* and recognized as hispidin (6-(3,4-dihydroxylstyryl)-4-hydroxy-2-pyrone) [13] — a well-known fungal and plant secondary metabolite [14] (Table 1). Further, hispidin was enzymatically converted into luciferin whose structure has been established as 3-hydroxyhispidin [13] (Fig. 1).

In the literature, there is a number of reports on isolation of hispidin and its derivatives from plants, including *Alpinia zerumbet* [15–17], *Pistacia atlantica* [18], *Peganum harmala* [19], *Pteris ensiformis Burm* [20], *Cassia alata* [21], *Rheum tataricum* [22] and others (Table 1). The wide occurrence of hispidin among fungal and plant species, along with the existence of similar compounds among plant metabolites have logically led us to an idea of challenging the possibility of engineering the self-luminescent plant by introducing a luciferase gene in a plant capable of biosynthesizing its substrate. Like other plant metabolites, hispidin derivatives are in the focus of modern investigations due to possible pharmaceutical applications. Hispidin and its derivatives are characterized by many bioactivities such as antioxidant, anti-cancer, anti-obesity and are regarded as potential leads for drug development [16–19].

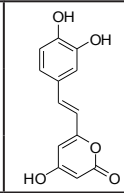
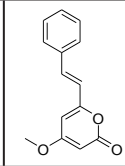
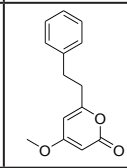
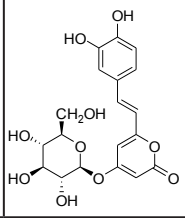
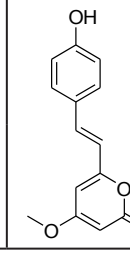
The purpose of this work was screening for the substrate(s) of fungal BL reaction in the extracts from the range of plants occurring in the European part of Russia and isolation of active compound(s).

MATERIALS AND METHODS

Plant extracts

All plant samples were collected in Tver region of Russia in June 2015 (Table 1 in SI). Only leaves were collected. All samples were frozen and stored at –70°C, some of them were dried *in vacuo.*

Table 1. Plants containing hispidin and similar compounds

Compound name	Structure	Species	References
Hispidin		*Alpinia zerumbet*	[15–17]
		Pistacia atlantica	[18]
		Peganum harmala	[19]
5,6-Dehydrokawain		*Alpinia zerumbet*	[15–17]
Dihydro-5,6-dehydrokawain		*Alpinia zerumbet*	[15–17]
Hispidin 4-O-β-D-glucopyranoside		*Pteris ensiformis Burm*	[20]
Bisnoryangonin		*Cassia alata*	[21]
		Rheum tataricum	[22]

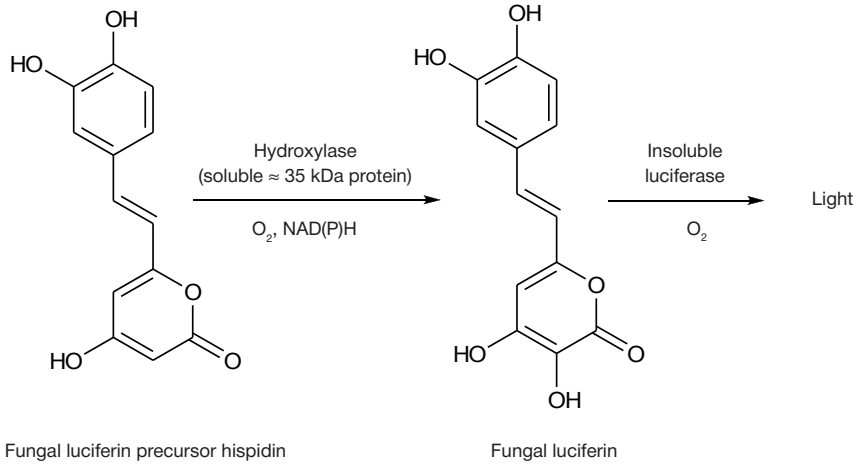


Fig 1. Fungal bioluminescence mechanism

Extraction of plant biomass is a well-designed technique [23]. Acetone was chosen for the initial screening of about 200 samples. Other solvents (methanol, ethanol, acetonitrile) were also tested for optimization of the extract conditions.

For preparation of a plant extract a piece of leave (30–500 mg) and 0.3–1.5 mL of a solvent were shaken in a 1.5 mL tube for 20 min on BioShake XP shaker at 1800 rpm at room temperature. The extract was centrifuged (5 °C, 10000 rcf on a 5424R Eppendorf centrifuge), and the solid residue was discarded. The extract could be further used for SPE or HPLC separation or, alternatively, dried in a vacuum centrifuge MiVac (SpScientific). For further operations dried residues were redissolved in an appropriate solvent and centrifuged. The solvent composition for the dried residue could be different from the solvent in the first extraction, in particular, a buffer solution was added to an organic solvent.

SPE technique with C18 pre-packed cartridges (500 mg, Phenomenex) was used: 0.5–1 mL of initial extract was eluted followed with 1 or 2 mL acetonitrile–water mixture 1 : 1, acetonitrile and acetone in this order.

Preparation of fungal luciferase is described in detail in our previous publication [13].

Bioluminescence assay

The bioluminescence assay comprised two components: fungal luciferase and plant organic extract. A diluted fungal enzymatic extract (3 µL) and a plant organic extract (3, 10 or 15 µl) were added to 100 µL of 0.2 M phosphate buffer, pH 7.5 with 1 mM DTT, in a test tube, immediately shaken and placed in a cuvette of a luminometer (Glomax 20/20, Promega). Luminescence was measured for 10 s with integration time 1 s (Table 2). Extracts from leaves, extracts of their dried residues of the first extracts, fractions after SPE or the fraction after HPLC were tested as organic extracts containing a potential substrate.

HPLC separations were performed on Nexera X2 (Shimadzu) instrument equipped with autosampler SIL-30AC, diode-array detector SPD-M20A and fraction collector FRC 10A. Columns of different types were applied (Table 3). Columns and detector were maintained at room temperature, autosampler temperature was 5°C. Mobile phase: buffer A — 0.02 M ammonium acetate pH 5.5, buffer B — acetonitrile or methanol or acetone (mobile phase composition and gradient see in Table 3). Solvents were of HPLC grade.

RESULTS

To discover a luciferin-like substrate in taxonomically diverse plants samples, the first task was a screening of the 200 species collection. Each plant extract was mixed with the fungal enzyme solution and luminescence was immediately measured. Totally, in 10 cases the luminescence (Table 4) was

Table 2. Samples for BL assay

Table/Figure	Sample preparation	Solvent/total volume, µL	Volume for assay, µL
Table 2	Frozen leaves 100 mg, extract	Acetone/300	3
Fig. 2	*P. natans* frozen leaves 100 mg, extract 300 µL in acetone dried and redissolved	AB solvent*/100	3
Fig. 3; 4, A, B blue	*R. nigrum* (A) and *B. pendula* (B) dried leaves 50 mg, extract	EtOH/1000 MeOH/1000	3 3
Fig. 3; 4, A, B red	SPE fractions of the same extracts	Acetone/1000	3
Fig. 3; 4, B	*B. pendula* leaves 100 mg (frozen) or 50 mg (dried), extract	MeOH/1000	10
Fig. 4	*B. pendula* HPLC fraction 500 µL dried and redissolved	MeOH/50	10
Fig. 5	*P. natans* HPLC fractions 1000 µL dried and redissolved	AB solvent/100	10
Fig. 6	*B. pendula* HPLC fraction of 1000 µL dried and redissolved	MeOH/50	10
Fig. 7	*P. natans* HPLC fractions 300 µL dried and redissolved	AB solvent/50	15
Fig. 8, A	*P. natans* HPLC 500 µL fractions	HPLC solvent/500	10
Fig. 8, B	HPLC fractions 500 µL	HPLC solvent/500	10

Note. * — AB binary solvent (A — acetone, B — 0.1 M NH_4Ac, pH 6.5).

Table 3. HPLC conditions

Figure	Column	Mobile phase*, component B	Sample
5	Discovery C18 5 µm 4.6 × 150 mm	MeCN grad 30–90 % 0–5 min, 90 % 5–10 min, flow 1 mL/min	*P. natans* frozen leaves, 300 mg, in 1 mL acetone, extract 500 µL dried and redissolved in 130 µL of AB solvent, 100 µL on column
6	ZORBAX SB-C18 5 µm 9.4 × 150 mm	MeOH, grad 60–100 % 0–6 min, 100 % 6–45 min, flow 2 mL/min	*B. pendula* fresh leaves, 400 mg in 1500 µL MeOH; SPE fractionated, 800 µL of active fraction in MeCN on column
7	TSK ODS-120T 5 µm 4.6 × 250 mm	Acetone grad 40–90 % 0–7 min, 90 % 7–30 min, flow 1 mL/min	*P. natans* dried leaves, 50 mg in 500 AB solvent, 200 µL on column
8, A	Lichrosorb Diol 10 µm 4.6 × 250 mm	MeCN grad 90–80 % 0–7 min, flow 1 mL/min	*P. natans* frozen leaves, 70 mg in 200 µL acetone, 100 µL on column
8, B	Synergi Polar RP 80A 4 µm 2 × 150 mm	MeCN grad 70–95 % 0–5 min, 95 % 5–14 min, flow 0.7 mL/min	Fraction 3–3.5 min of the first chromatography concentrated to 100 µL

Note. * — Component A — 0.02 M NH_4Ac, pH 5.5.

Table 4. BL activity of plant extracts

Plant	Extract from frozen leaves, luminescence, rel. units	Dried and redissolved extract, total luminescence		
		Compared to initial extract, %	After exposing at 20 °C for 2 h compared to before exposure, %	After exposing at 0 °C for 2 h compared to before exposure, %
Andromeda polifolia	6 600	30	35	no data
Betula pendula	11 500	25	40	90
Chamardaphne calyculata	7 900	35	40	120
Potamugeton natans	290 000	25	6	150
Pyrola rotundifolia	1 500	55	70	130
Ribes nigrum	11 000	40	30	330
Ribes rubrum	8 500	35	20	140
Salix aurita	11 000	20	15	no data
Salix pentandra	10 000	30	15	60
Stachys sylvatica	11 000	20	25	no data

observed. For the initial screening we used only acetone for extraction. The composition of the solvents varied to achieve the maximum luminescence of the extracts with activity found. As a result, some quantitative, but not qualitative differences in BL values were observed. Drying the extract and redissolving of the residue in a smaller volume allowed to concentrate the sample and to increase the BL values. A buffer solution with a certain pH value was added to the organic solvent in some cases, the pH value being critical for the preservation of the extract activity. Thus, the following conditions were the best for frozen leaves of *P. natans*: acetone extraction from leaves material (first extraction) and with mixture 7 : 3 of acetone and aqueous acetate buffer pH 6.5 for redissolving the dried residue (Fig. 2).

The extracts of all active species were tested for stability. They were dried and dissolved again, then exposed for some hours at 0 °C or at room temperature. Measurements of BL activity after each operation demonstrated that all the substrates were unstable (Table 4). Loss of activity during the drying process was 50 % and more. The highest activity (observed for *P. natans* extract) decreased by an order after exposure at room temperature.

When exposing the extracts on ice evident increase of luminescence was observed during some hours. This effect was noticed for many species, and particularly a 10x increase was observed for *R. nigrum* extracts (Table 4, Fig. 3, A). The dependency of activity on the exposure time varied not only between species but also between initial extracts and SPE fractions of the extracts. The extracts from dried leaves of B. pendula seemed to be stable (Fig. 3, B), while the extracts from fresh and frozen leaves showed a significant growth of BL (Fig. 3, C). Furthermore, 10-s curves of the BL measurments for *B. pendula* extracts were often (but not always) of rising type instead of a usually observed declining type (Fig. 4).

Based on the highest value of initial luminescence, we chose *P. natans* as a first candidate to continue with HPLC separation of the BL substrate. Mobile phase conditions for HPLC separation of the extracts were optimized as follows: buffer A 0.02 M aqueous acetate buffer at pH 5.5, elution with gradient buffer B content up to 95 %. HPLC fractions were dried *in vacuo*, dissolved again with 20 times concentration and their luminescence activities were measured (Fig. 5). We observed two zones in the HPLC profile, which comprised active components, along with many components with different UV spectra, indicating that the extract contained more than one substrate and the components were not completely separated.

Looking for isolation of higher amounts of the unstable substrates we varied our technique and tried other species. Fig. 6 represents the results of these attempts. *B. pendula* was chosen as a source. Extract of fresh leaves (400 mg in 1500 µL of methanol, 1000 µL of extract taken) was fractionated on C18 cartridges. Activities of the fractions were measured and 800 µL of the most active fraction was loaded without drying onto a semi-preparative C18 column. Assaying BL activity of the fractions revealed many active zones on a 45 min chromatogram. It can be mentioned, that green color (absorption around 650 nm) always accompanied the activity. We could ascertain the presence of many unresolved peaks in the most active fractions at the beginning of the chromatograms, some active fractions at the end and the absence of correlation of major chromatographic peaks with activity. Second step separation of some fractions on the same column was performed, but activities disappeared due to chemical instability.

In another attempt we used acetone as an unusual mobile phase component. Acetone is not very suitable for UV detection in lower UV region (300 nm and less). However, we supposed that active BL substrates should probably absorb in the region above 300 nm, making possible the use of acetone. The chromatogram and the activity profile are shown on Fig. 7. The profile indicated the presence of several active components. The activities and the amounts of active substances were not high enough to perform the next chromatographic step.

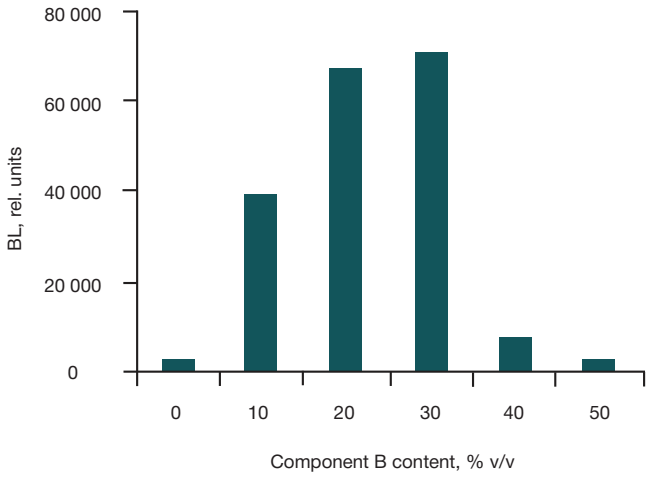


Fig. 2. Dependence of BL (rel. units) on component B content (% v/v) in binary solvent (A — acetone, B — 0.1 M NH_4Ac, pH 6.5) for redissoving of *P. natans* dried extract

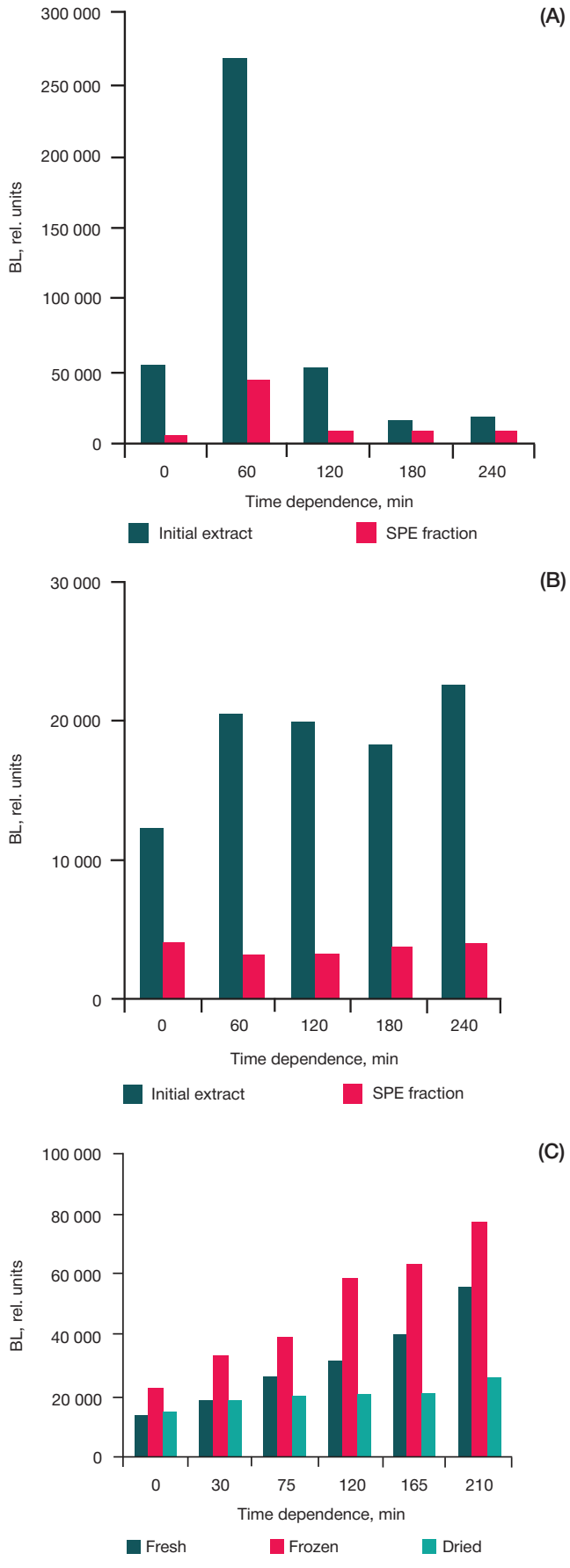


Fig. 3. Time dependence (min) of BL (rel. units) of leaves extracts and SPE fraction of the extracts (exposure at 0 °C): *R. nigrum* **(A)**; *B. pendula* **(B)**; extracts of different type material of *B. pendula* leaves **(C)**

In another attempt we applied faster separation of *P. natans* extracts on a polar column as a first step combined with separation of the most active fraction on a reverse phase column as a second step (Fig. 8). To prevent activity losses extracts and fractions were not dried before BL measurements. The most active 500 µL fraction (between 3 and 3.5 min) from the first column was concentrated to 100 µL and loaded onto the second column. We could reveal some active fractions after the second separation. Two peaks were distinguished on the cromatogram in the region of 11.5 and 12.0 min but separation was not complete and the activities were low.

We estimated the losses of the activity at each stage of the separation by BL measurement (Table 5). Total activity of the solutions was calculated for initial extracts and fractions after separations. These data showed that the losses occurred at each operation. As a result, only 0.2 % of the initial activity remained after final step. Thus, in all cases activity losses during separation did not allow us to isolate any individual substance for further characterization.

DISCUSSION

The study of 10 plants containing BL active compounds showed that our hypothesis about the presence of hispidin or 3-hydroxyhispidine in the leaves samples was wrong. In all cases NAD(P)H was unnecessary for the luminescence reaction, indicating similarity of the active compound to fungal luciferin, but not to hispidin. We applied the same chromatography conditions as those previously developed for isolation of the fungal luciferin [13]. However, activity profiling of HPLC fractions showed no activity and therefore the absence of 3-hydroxyhispidin.

The presence of many active fractions after chromatographic separation of the organic extracts indicates that each plant synthesizes not one but many substrates for the fungal luciferase. In addition, a variety of chromatographic conditions for separation of different plants extracts showed the diversity of their active compound sets. Anomalous increase of bioluminescent activity during exposure of organic extracts on ice suggests the possibility of chemical reactions resulting in decrease of some active compounds concentration and increase in the concentration of others. Unfortunately, no

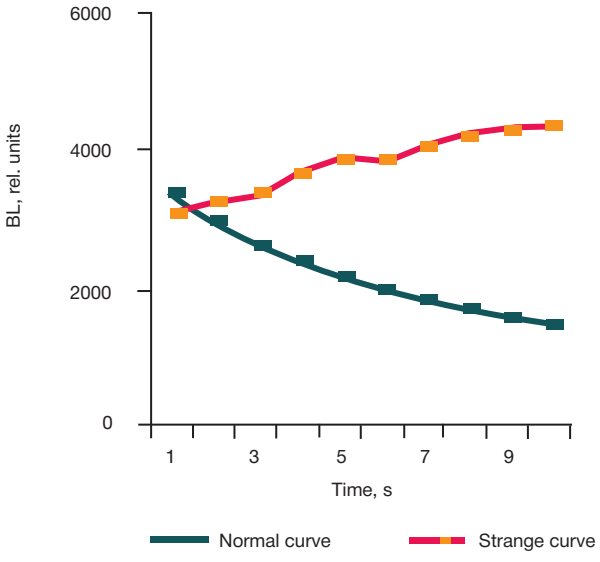
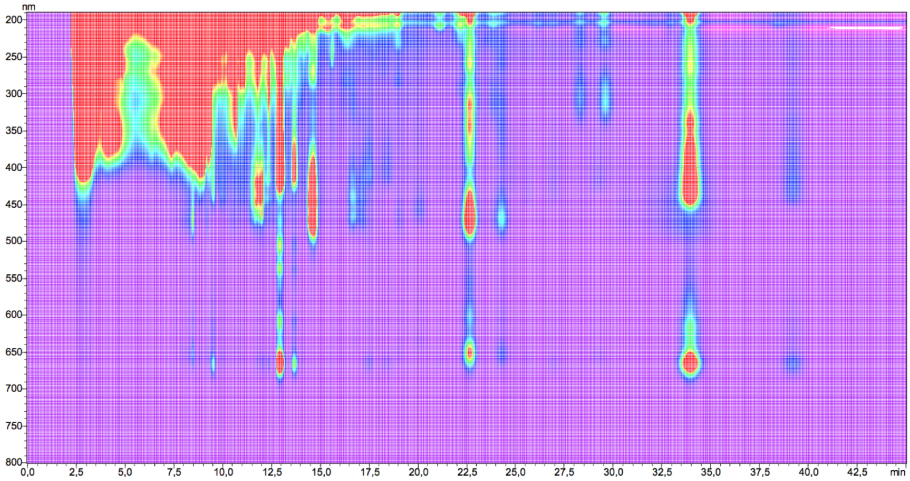


Fig. 4. Two types of 10 s BL assay curves of *B. pendula* HPLC fractions

Fig. 5. HPLC separation of *P. natans* extract: chromatogram (UV 430 nm, blue line), luminescence of fractions (red line) and mobile phase composition (light blue line). All other conditions see Tables 2 and 3

(A)



(B)

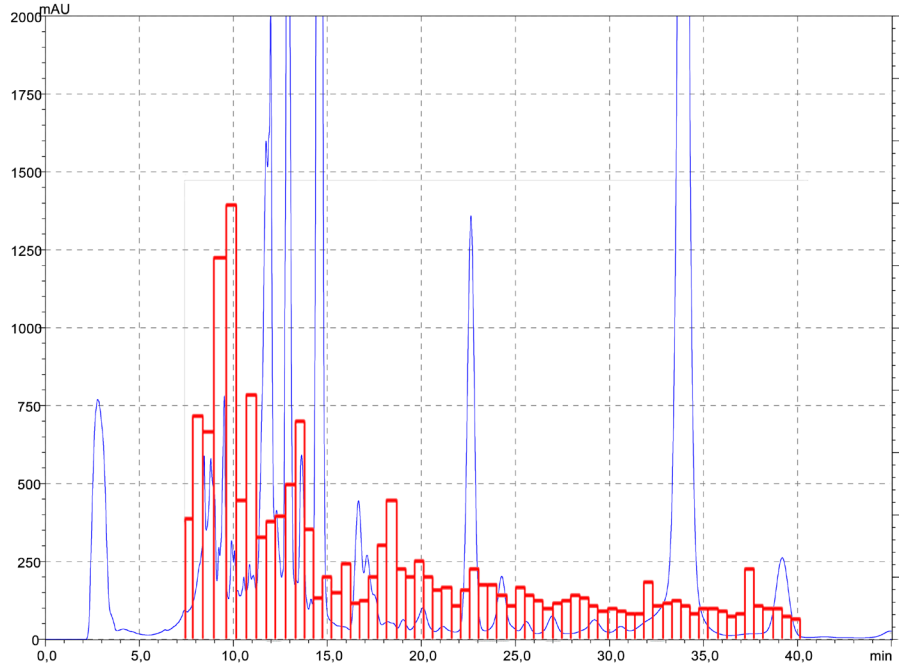


Fig. 6. HPLC separation and luminescence of *B. pendula* extract: **(A)** — UV profile; **(B)** — chromatogram (UV 430 nm, blue) and activity profile (red). All conditions see Tables 2 and 3

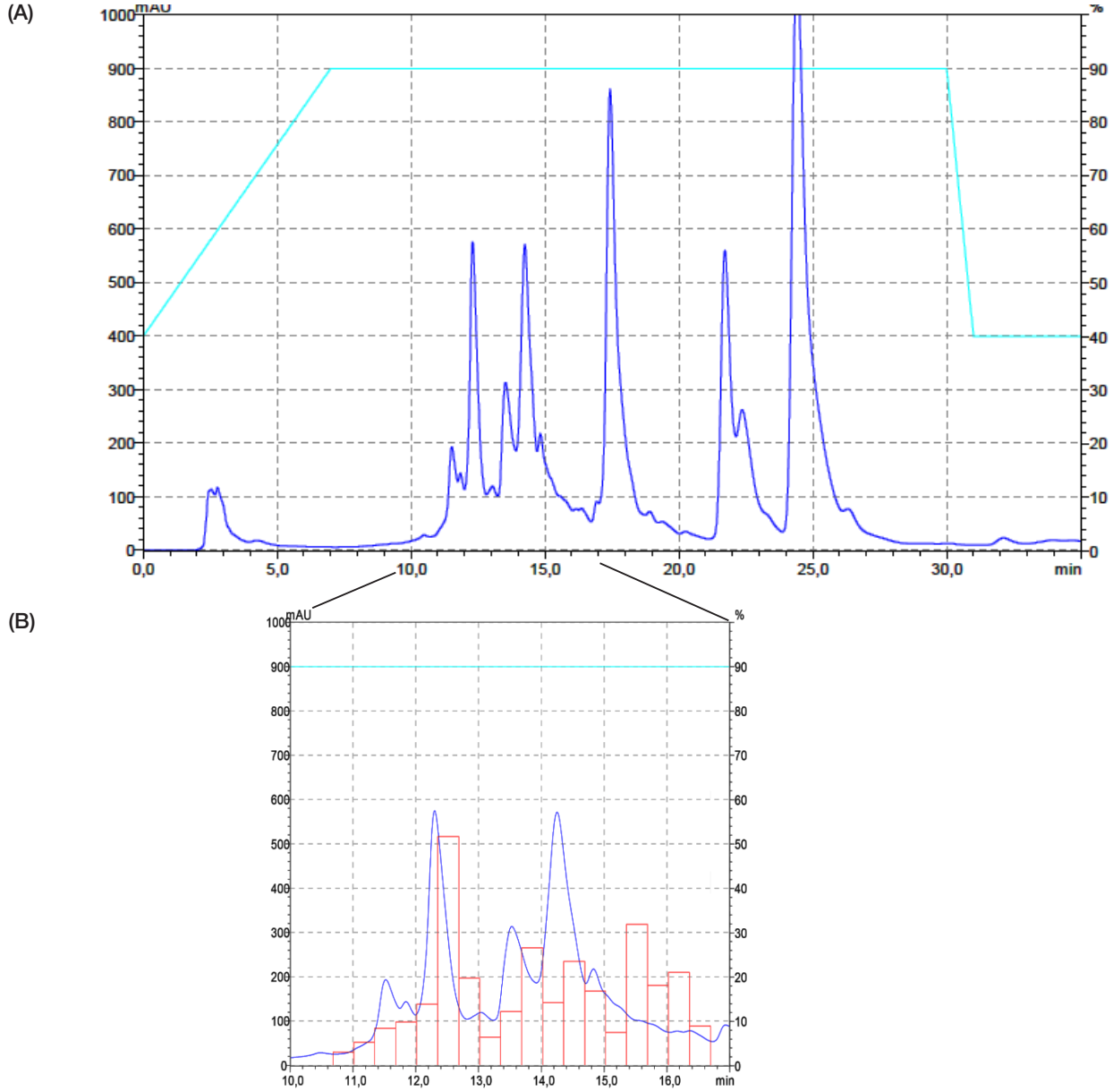


Fig. 7. HPLC separation of *P. natans* extract with acetone as a mobile phase component and luminescence profile: **(A)** — chromatogram (UV 430 nm, blue line) and mobile phase composition (light blue line), **(B)** — region of 10–17 min of the chromatogram (blue line), luminescence of fractions (red line). All conditions see Tables 2 and 3

stable substrates could be detected, as the luminescence of the extracts of all 10 plants decreased at room temperature.

We have applied many modifications for organic extracts preparation and their HPLC separation to satisfy the contradictory requirements of maximum recovery, maximum stability and selective isolation of the individual unstable compound(s) from complex mixtures. Some extracts were fractionated with SPE prior to HPLC, and the others were immediately loaded onto the chromatography column. We tried to dry the extracts for additional concentration, the same was applied to the solutions of the chromatographic fractions before measuring luminescence. Various organic solvents were used as mobile phase components for HPLC; two-dimensional chromatography was performed on columns with different polarity.

But ultimately, the stability of all the active compounds found was insufficient to achieve the substrate isolation in an amount sufficient to establish the compounds' structure and properties.

CONCLUSIONS

Bioluminescent plants do not exist in nature, making their creation by the means of synthetic biology a challenge. This research has revealed the ability of some plant components to react with fungal luciferase to produce luminescence. Thus, the present study may be considered as a first step to creation of self-luminescent plants. Within the species range covered in this work, all potential substrates were chemically unstable, making their isolation and structural characterization yet unsuccessful. However, the following conclusions may be considered in the future attempts: the luminescent luciferase substrates present in the plant biomass extracts are not identical to fungal luciferin (3-hydroxyhispidin); the species tested produce many different active substances. Probably, future work towards self-consistent bioluminescent plants should focus on finding the genes responsible of 3-hydroxyhispidin biosynthesis and expression of these genes in a transgenic plant together with fungal luciferase.

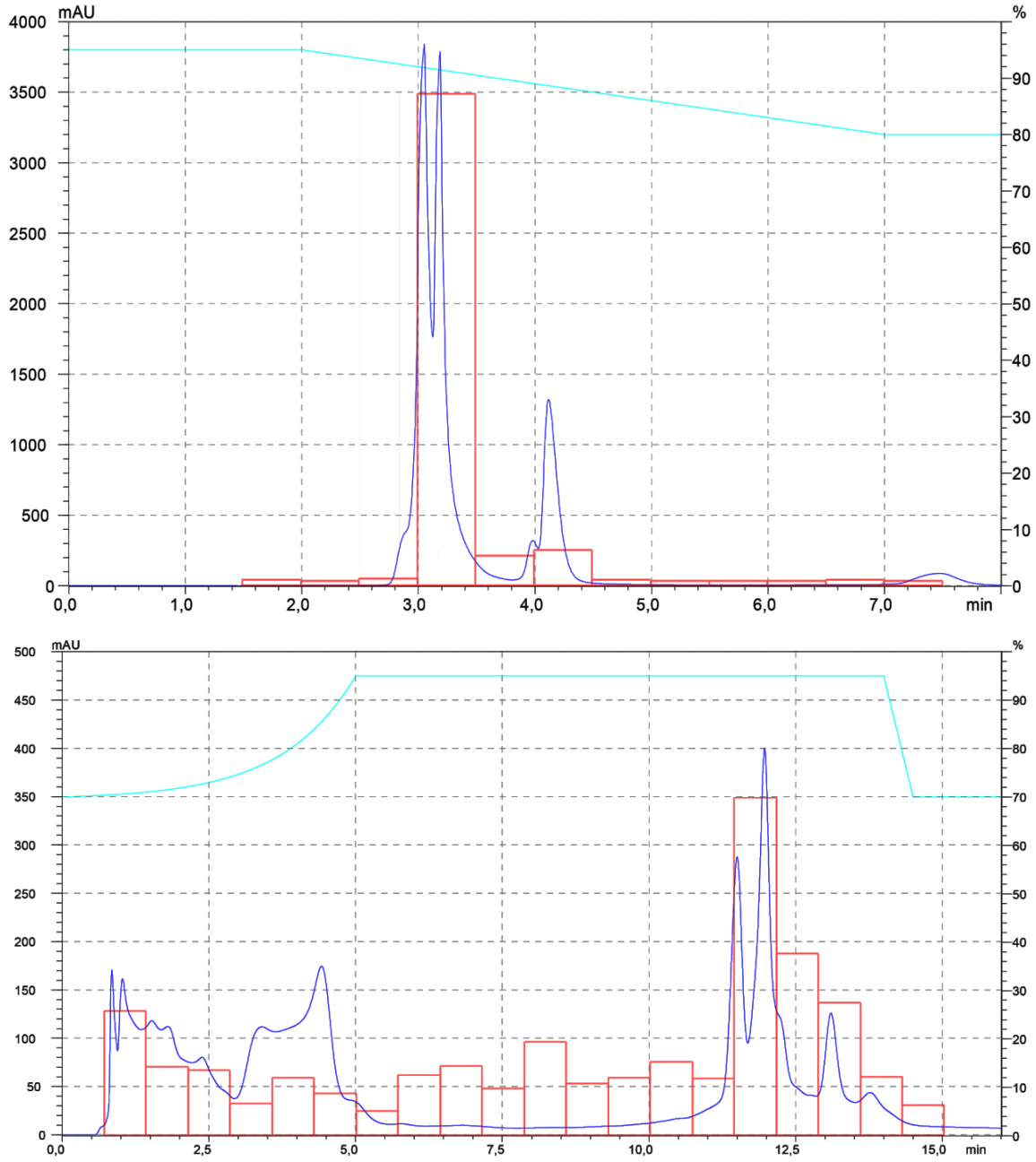


Fig. 8. Two dimensional HPLC separation of *P. natans* extract and luminescence profile: chromatograms (UV 430 nm, blue lines), activities profile (red lines) and mobile phase compositions (light blue line): **(A)** — diol column, **(B)** — RP column. All other conditions see in Tables 2 and 3

Table 5. Losses of activity in separation process (see Fig. 8)

Stage	Activity *a*, rel. units	V_1/V_2*	Total activity *A***, rel. units	Total activity compared to the previous step, %	Total activity compared to initial, %
Extraction	280 000	100/1	28 000 000	100	100
Chromatography #1	52 000	500/10	260 000	9	9
Effect of fractionation	45 000	500/10	230 000	87	8
Drying	27 000	100/3	890 000	39	3
Chromatography #2	6 500	500/10	33 000	37	1
Effect of fractionation	1 200	500/10	59 000	18	0,2

Note. * — V_1 — total solution volume; V_2 — solution volume in measurement; ** — $A = a \cdot V_1/V_2$.

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