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Dear Colleagues,

Here is the first issue of the volume 31 of Acta Microbiologica Bulgarica. After the publication of volume 30th, a break of more than 20 years followed, due to the individual striving and the requirement of the research organizations in our country for publishing in international journals possessing impact factor. Besides, the economic situation reflected strongly on the science in this country. Nevertheless, the development of our science continued. Moreover, we initiated the establishment of the Balkan Society for Microbiology (BSM) in 1998. This Society became stronger by embracing microbiological societies of Serbia, Turkey, Greece, Romania, Montenegro, Albania, Bosnia and Herzegovina, FYROM and Bulgaria and became an important factor for the development of microbiology in the South-Eastern Europe. The main activity of BSM was congresses, Microbiologia Balkanica, each two years. Two of them were carried in Bulgaria, the First in Plovdiv (1999) and the Eight in Veliko Tarnovo (2013). Thessaloniki will welcome delegates of the consecutive 9th congress this year.

Bulgarian Society for Microbiology (BgSM) was established in 1927, in 1952 being included in the Union of Scientists in Bulgaria. It carried out regularly national congresses with a wide international participation every 4 years. The last, 13th Congress was held in Tryavna in October 2014. Actually, more than 200 Bulgarian microbiologists are members of BgSM. The idea AMB to be restored as an official of the BgSM gave rise within the society members as a result of their desire to have a national scientific journal. This journal will unite the research attainments in all branches of the science microbiology: general microbiology, medical, veterinary, plant and soil microbiology, virology, mycology, industrial (applied) microbiology, food microbiology and infectious immunology.

The journal will be edited in English. Its Editorial Board includes leading Bulgarian scientists and a series of pronounced in the scientific community foreign scientists. The strong presentation of the achievements of Bulgarian scientists as well as their publishing activity are guarantee for the success of the Journal.

Let wish a tail-wind and a success of Acta Microbiologica Bulgarica!

Editor-in-Chief

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Review**Antibiotic Resistance - a World Challenge**

Encho Savov*, Angelina Trifonova, Ivanka Gergova, Maya Borisova, Elena Kjoseva, Iva Todorova

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Abstract

Antibiotic resistance is a worldwide health problem that continues to increase. The European Union and World Health Organization declared the rapid development of antimicrobial resistance as one of the three greatest threats to human health. In this paper are presented current data from literature and data of our own, concerning the increasing antibiotic resistance in the world. There was registered a significant increase in the proportion of multiresistant bacteria to the main groups of antimicrobials used in the clinical practice - third-generation of cephalosporins, carbapenems, quinolones, and aminoglycosides. Among the so-called multidrug-resistant or pandrug-resistant bacteria are *Acinetobacter baumannii*, methicillin-resistant *Staphylococcus aureus* (MRSA, multi-drug resistant), extended-spectrum beta-lactamase (ESBL) producing *Klebsiella* species and *Escherichia coli*, and others. Indication for the significance of the problem in military settings is the determination of an increase in the number of reported multidrug-resistant *A. baumannii* in bloodstream and wound infections in US soldiers at military medical facilities in Iraq, Kuwait, and Afghanistan. In this context, at the EU-US Summit in November 2009 were made decisions and developed strategies, which could be better addressed by cooperation between the United States and Europe for improving of the use of antibacterial drugs.

Key words: resistance, multidrug-resistant bacteria, ESBL-producing bacteria, MRSA, treatment

Резюме

Резистентността към антибиотици е световен здравен проблем. Специалистите от Европейския съюз (ЕС) и Световната здравна организация (СЗО) декларираат, че бързото развитие на резистентност към антимикробни средства е една от трите най-големи заплахи за човешкото здраве. В настоящата работа представяме съвременни литературни и собствени данни за нарастващата резистентност на проблемни за болничната патология микроорганизми към антибиотици и химиотерапевтици. Регистрирани са значителен брой микроорганизми, резистентни към основни групи антимикробни средства, използвани понастоящем в клиничната практика като трета генерация цефалоспорици, карбапенеми, хинолонови производни и аминогликозиди. Специално внимание заслужават т.н. множествено-резистентни бактерии, включващи *Acinetobacter baumannii*, метицилин-резистентни *Staphylococcus aureus* (MRSA), множествено-резистентни щамове *Klebsiella pneumoniae* и *Escherichia coli*, продуциращи широко-спектърни бета-лактамази (ESBL) и карбапенемази. Индикация за значимостта на проблема за военната медицина, е и нарастването броя на съобщенията, свързани с изолирането на множествено-резистентни щамове *A. baumannii* като причинители на септични състояния и раневи инфекции в американски войници от мисии в Ирак, Кувейт и Афганистан. В този смисъл, на среща на „върха“ през 2009 г. между представители на Европейския съюз и САЩ, се взема решение за бъдещи стратегии, насочени към коопериране усилията на специалистите за оптимизиране борбата с феномена антимикробна резистентност.

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The era of antibiotics is drawing to a close. In just a couple of generations, what once appeared to be miracle medicines, have been beaten into ineffectiveness by the bacteria, they were developed to knock out. Once, scientists hailed the end of infectious diseases. Now, the post-antibiotic apocalyptic is within sight. Sarah Boseley, The Guardian, UK. In 2009, the World Health Organization (WHO) declared that antibiotic resistance is one of the greatest threats to health on a global scale (Kaplan *et al.*, 2013).

Introduction

Antibiotic resistance is a worldwide public health problem that continues to grow. When penicillin became widely available during the World War II, it was a medical miracle, rapidly vanquishing the biggest wartime killer - infected wounds. But just four years after drug companies began mass-production of penicillin in 1943, microbes began to appear in a way that could resist it. The first microorganism to battle penicillin was *Staphylococcus aureus* (Lewis, 1995). Another type of penicillin resistant pneumonia, caused by *Streptococcus pneumoniae* surfaced in a remote village in Papua New Guinea in 1967. American military personnel in Southeast Asia were acquiring penicillin-resistant gonorrhea from prostitutes (Lewis, 1995) at about the same time. A hospital-acquired intestinal infection caused by the bacterium *Enterococcus faecium* joined the list of microorganisms that outwit penicillin in 1983. At present, resistance increased to a number of commonly used antibiotics. We have come to point certain infections (*Acinetobacter baumannii* infections) in the 1990s, that we do not have agents available for. According to the report in the New England Journal of Medicine from April 28, 1994, researchers have identified bacteria in patient samples that resist all currently available antibiotic drugs (Lewis, 1995). The data from the European Union shows that 25 000 deaths per year were attributed to infections caused by antibiotic-resistant bacteria, of which 66% were Gram (-) bacilli. The total number of additional hospital days required for treatment of resistant bacteria is 2.5 million days per year at a cost of 1.5 billion euros (www.medscape.com/viewarticle/717606-3).

The aim of this study is to present current data about the resistance and multiresistance of bacteria, which are problematic for human health, to antimicrobials, their behavior in the infections and the strategies for improving of their treatment.

Bacterial Resistance Weaponry

The most serious, life-threatening infections are caused by a group of drug-resistant bacteria that the Infectious Diseases Society of America (IDSA) has labeled the „ESKAPE“ pathogens, because they effectively escape the effects of antibacterial drugs. Table 1. (<http://www.medscape.com>)

Enterococcus sp. infections

Enterococci are intrinsically resistant to a broad range of antibiotics including cephalosporins, penicillins, sulfonamides, and low concentration of aminoglycosides. Based on our data (Fig.1), as a possibility for treatment of infections, caused by *E. faecalis*, continue to be vancomycin with 0.4% resistance for 2008, teicoplanin - 1.3%, linezolid - 2.4%, and a combination of ampicillin/sulbactam - 0.22% (Savov *et al.*, 2010).

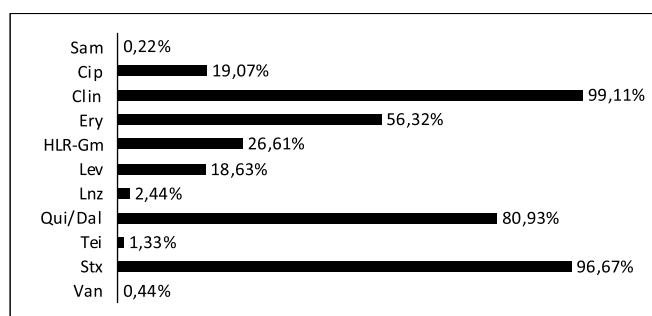


Fig.1. Resistance of *E. faecalis* to antimicrobials (n=455) - 2008

Van - vancomycin, Stx - sulfam/trimet, Tei - teicoplanin, Qui/Dal - quinopristin/dalfopristin, Lnz - linezolid, Lev - levofloxacin, Ery - erythromycin, Clin - clindamycin, Cip - ciprofloxacin, Sam - ampicillin/sulbactam, HLR-Gm - high level resistance to gentamicin

Particularly virulent strains of *Enterococcus* that are resistant to vancomycin (vancomycin-resistant *Enterococcus* or VRE) have emerged in nosocomial infections of hospitalized patients especially in the US in the last two decades. (Fisher *et al.* 2009). Other developed countries such as the UK have been spared of this epidemic, and Singapore managed to halt an epidemic of VRE in 2005. VRE may be treated with quinopristin/dalfopristin (Synercid) with response rates of approximately 70% (Tunger *et al.*, 2004).

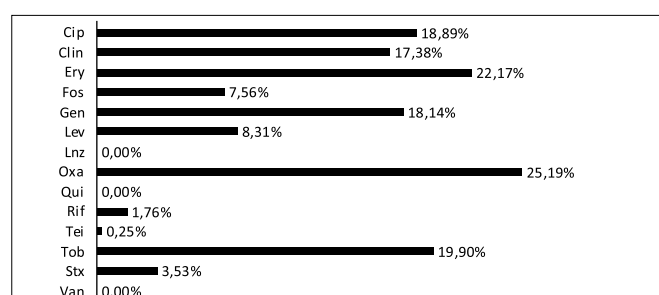
Methicillin-Resistant *Staphylococcus aureus* (MRSA)

Reports of methicillin-resistant *Staphylococcus aureus* (MRSA) - a potentially dangerous type of *Staphylococcus* bacteria that is resistant to certain antibiotics and may cause skin and other infections - in persons with no links to healthcare

Table 1. The most serious life-threatening infections

E	<i>Enterococcus faecium</i>	Third most common cause of healthcare-associated HCA BSI. Increasing resistance to vancomycin.
S	<i>Staphylococcus aureus</i> (MRSA)	Emerging resistance to current drugs and significant drug toxicities. Lack of oral agents for step-down therapy.
K	<i>Klebsiella</i> <i>Escherichia coli</i> <i>K. pneumoniae</i>	ESBL-producing organisms increasing in frequency and severity; associated with increasing mortality. <i>K. pneumoniae</i> carbapenemases causing severe infections in LTCF. Few active agents; nothing in development.
A	<i>Acinetobacter baumannii</i>	Increasing worldwide, recent surge reported in hospitals. Very high mortality. Carbapenem-resistant.
P	<i>Pseudomonas aeruginosa</i>	Increasing <i>P. aeruginosa</i> infections in the US and worldwide. Resistant to carbapenems, quinolones, aminoglycosides.
E	<i>Enterobacter</i> species	MDR HCA infections increasing; resistance via ESBLs, carbapenemases, and cephalosporinases.

HCA = healthcare associated; BSI = bloodstream infection; MRSA = methicillin resistant *S. aureus*; ESBL = extended-spectrum beta-lactamase; LTCF = long-term care facility; MDR = multiple drug-resistant

**Fig. 2.** Resistance of *S. aureus* to antimicrobials (n=397) - 2008

Van - vancomycin, Stx - sulfam/trimet, Tob - tobramycin, Tei - teicoplanin, Rif - rifamycin, Qui/Dal - quinopristin/dalfopristin, Oxa - oxacillin, Lnz - linezolid, Lev - levofloxacin, Gen - gentamicin, Fos - fosfomicin, Ery - erythromycin, Clin - clindamycin, Cip - ciprofloxacin

systems, have been observed with increasing frequency in the US and elsewhere around the globe.

The relative part of infections caused by MRSA varies in the different countries. According to Jewell, M. (Jewel, 1994), this type of infections is a big problem in Chicago hospitals, where the number of MRSA infections is about 60%. In Spain and in France this number is between 30-33%. (Herwaldt *et al.*, 1995). The data for the Military Medical Academy in Sofia, Bulgaria showed that about 25% of the *S. aureus* infections were caused by MRSA (Fig.2) (Savov *et al.*, 2010). The options for treatment of this type of infections at the moment are: vancomycin, teicoplanin, linezolid, and tigecyclin.

Multidrug-Resistant *E. coli* and *Klebsiella* sp.

Multi-drug resistant, extended-spectrum beta-lactamases (ESBL) producing *Klebsiella* species and *Escherichia coli* have been isolated

in hospitals throughout the world. ESBL positive strains are associated with increased mortality, because of the failure to treat infections, caused by ESBL positive organisms, due to the limited therapeutic choices (Kim *et al.*, 2002; Paterson *et al.*, 2005). Of all “EARSS-specific” pathogens, *E. coli* demonstrated the most worrying trends. *E. coli* isolates with multiple resistance to third-generation cephalosporins, fluoroquinolones, and aminoglycosides were registered in Bulgaria in great proportions (EARSS 2002). The relative part of the *E. coli* strains, producing ESBL in the units of Military Medical Academy (MMA) in the last 3 years is about 20%. As compared to 2007, the relative part of *K. pneumoniae* strains, producing ESBL significantly increased from 53% in 2007 up to 67.8% in 2008 (Savov *et al.*, 2010). The results, presented in an extensive study performed in Bulgaria for a period of eight years (1996 - 2003), showed, that the most widespread enzymes found in *Enterobacteriaceae* belong to three groups - SHV-12, CTX-M-15, and CTX-M-3 as well (Markovska *et al.*, 2008). The possibility for treatment of such of infections stay only carbapenems and beta-lactams in combination with beta-lactamase inhibitors.

Multidrug (Pandrug) Resistant *Acinetobacter baumannii* Infections

Acinetobacter baumannii has emerged as one of the most troublesome pathogens for health care institutions on a global scale. Its clinical significance, especially over the last 15 years, has been propelled by its remarkable ability to acquire resistance determinants, making it one of the organisms threatening the current antibiotic era

(Davis *et al.*, 2005). The rapid global emergence of *A. baumannii* strains resistant to all β -lactams, including carbapenems, quinolones and other antimicrobials illustrates the potential of this organism to respond swiftly to changes in selective environmental pressure (Peleg *et al.*, 2008). After performing whole-genome sequencing of a clinical epidemic *A. baumannii* strain found in France (AYE), an 86-kb resistance island, one of the largest to be described thus far, was identified (AbaR1). Overall, 52 resistance genes were identified, and surprisingly, 45 (86.5%) were localized in the AbaR1 resistance island (Fournier *et al.*, 2006). The resistance to carbapenems at MMA in Sofia (85% to meropenem) (Fig. 3) is associated with the production of Oxa 23 and Oxa 58 carbapenemases, but not to metallo-beta-lactamases (Stoeva *et al.* 2009; Savov *et al.* 2010).

The resistance to quinolones was assessed at the DNA level for mutation detection in quinolone-resistance-determining regions (QRDRs) and the subsequent aminoacid substitution in the GyrA and/or the ParC enzymes. A strong correlation was found between quinolone resistance and mutations in gyrA codon 83 and/or in the parC gene (codons 80 or 84) (Deccache *et al.*, 2011).

Additionally, there is an indication of an increase in the number of reported *A. baumannii* bloodstream infections in soldiers at military medical facilities in Iraq, Kuwait, and Afganistan. Fifty-three percent of *A. baumannii* infections have been registered as a bloodstream infections at the Walter Reed Army Medical Center /WRAMC/, which is the major US site receiving casualties from the conflict in Iraq/Kuwait and in Afganistan (Hawley *et al.*, 2007; Hujer *et al.*, 2006).

The choice of antibacterials for treatment of such of infections is difficult. The most promising data with regard to *A. baumannii* are the benefits of a prolonged infusion of up to 3 hours and increasing of the dose of up to 2 g per every 8 hours of meropenem administration; the concentration, obtained by this way in serum of above 16 μ g/ml for almost 60% of the time, supports the use of an extended meropenem infusion time for treating serious *A. baumannii* infections (Peleg *et al.*, 2008). The use of the polymyxins and tigecycline is very important for the treatment of serious infections with multidrug-resistant *A. baumannii*, however the Food and Drug Administration (FDA), the Clinical and Laboratory Standard Institute (CLSI), and the European Committee on Antimicrobial Susceptibility Testing (EUCAST) have established no breakpoints

for interpretation of antibiotic susceptibility testing of tigecycline against *A. baumannii*. These data suggest that caution should be taken in considering tigecycline treatment for *A. baumannii* infection in sites where drug levels may be suboptimal, such as the bloodstream (Peleg *et al.*, 2008). Unlike EUCAST and the British Society for Antimicrobial Chemotherapy (BSAC), the CLSI has established breakpoints for colistin and polymyxin B against *A. baumannii* (Peleg *et al.*, 2008). Clinical use of polymyxins against *A. baumannii* isolates proved to be extremely successful (Neonakis *et al.*, 2011). These antimicrobials have been tested extensively in combination with other agents against multiple drug-resistant *A. baumannii* - carbapenems, cefepime, amikacin, and others. Clinically, the combination of colistin with meropenem appears to be superior to the other agents (Neonakis *et al.*, 2011).

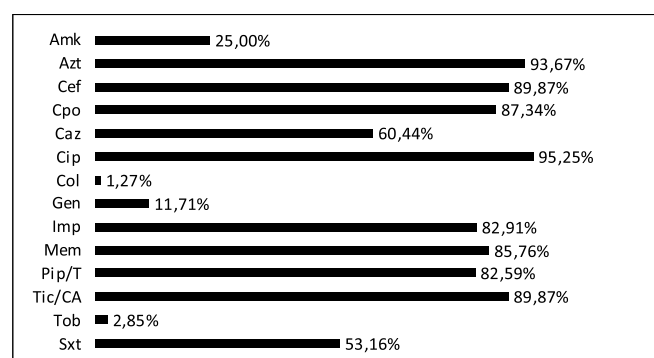


Fig. 3. Resistance of *A. baumannii* to antimicrobials (n-530) - 2008

Sxt - sulfam/trimet, Tob - tobramycin, Pip/Taz - piperacilin/ tazobactam, Mem - meropenem, Imp - imipenem, Gen - gentamicin, Col - colistin, Cip - ciprofloxacin, Caz - ceftazidim, Cef - cefepim, Azt - aztreonam, Amk - amikacin, Cpo - ceftiprom

Multidrug-Resistant *Pseudomonas aeruginosa* Infections

The Gram (-) bacterium *Pseudomonas aeruginosa* is a significant opportunistic pathogen. Chronic infections due to this organism are prevalent in cystic fibrosis patients and are frequently recalcitrant to treatment. In addition to displaying high levels of intrinsic antibiotic resistance, *P. aeruginosa* frequently converts to a mucoid state resulting in a rapid adaptive resistance that accounts for the high failure rate of antibiotic therapy in eradicating these infections. *P. aeruginosa* is intrinsically resistant to the majority of antimicrobial compounds due to its selective ability to exclude various molecules from penetrating its outer membrane. It is a problem, because in many cases the *P. aeruginosa* strains isolated were multiresistant (with resistance to: piperacillin/tazobactam of 22.7%, cefepime - 51.5%, and ceftazidime -

46.9%). The level of the resistance to carbapenems is about 32.2% for imipenem and also 41.7% for meropenem (Fig. 4) (Savov *et al.*, 2010).

The spread of similar multiresistant strains is very important for big hospital complexes according also to Edalucci *et al.*, 2008. This multiresistance usually is connected with production of metallo-beta-lactamase (MBL) VIM-2 and also these widespread clones, responsible for human infections, belong to O11 and O12 serotypes (Edalucci *et al.*, 2008).

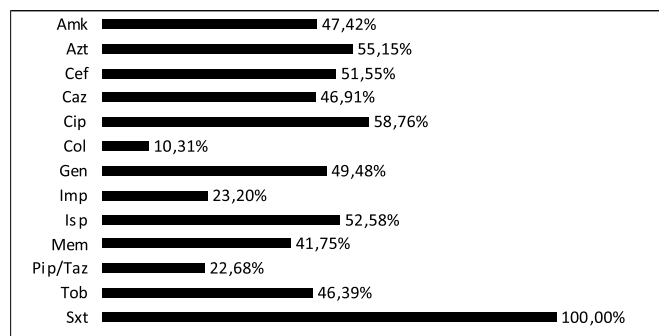


Fig. 4. Resistance of *P. aeruginosa* to antimicrobials (n=277) - 2008

Sxt - sulfam/trimet, Tob - tobramycin, Pip/Taz - piperacilin/tazobactam, Mem - meropenem, Isp - isepamycin, Imp - imipenem, Gen - gentamicin, Col - colistin, Cip - ciprofloxacin, Caz - ceftazidim, Cef - cefepim, Azt - aztreonam, Amk - amikacin

Resistance to ciprofloxacin and aminoglycosides is also high - 58.8% and 47-49% respectively (Fig. 4). (Savov *et al.*, 2010).

Multidrug-Resistant Bacteria Linked with New Delhi Metallo-Beta-Lactamase 1 (NDM-1)

A novel enzyme, described during 2009 - NDM-1 (New Delhi Metallo-Beta-Lactamase 1) (Yong *et al.*, 2009), which is encoded by blaNDM-1 gene, is increasingly dominant (Kumarasamy *et al.* 2010, www.phac-aspc.gc.ca)

The NDM-1 gene produces an enzyme which makes bacteria resistant to most antibiotics (fluoroquinolones, aminoglycosides and beta-lactams, including carbapenems (imipenem, meropenem, ertapenem, doripenem), except tigecycline and colistin. Carbapenems are powerful, broad-spectrum antibiotics, which are often considered to be the last line of defence against multi-resistant strains of bacteria, such as *E. coli* and *K. pneumoniae*. NDM-1 is strongly linked to India and Pakistan and many of the UK cases have recent medical exposure in the Indian-subcontinent (Kumarasamy *et al.*, 2010). All of the 21 UK producers comprise *K. pneumoniae* (14), *E. coli* (4), *Enterobacter spp.* (1), and *C. freundii* (2) from 18

patients and 16 hospitals scattered across England, and also one in Scotland. Forty strains with NDM-1 were isolated for 2009. In the US, three cases have been confirmed - in California, Illinois, and Massachusetts. Researchers believe all three patients picked up the resistant microorganism in hospitals in India (Kumarasamy *et al.*, 2010). The first positive for NDM-1 eleven local *E.coli* strains, proven by real time polymerase chain reaction (PCR) NDM-1 kit were isolated from clinical samples at the Military Medical Academy in Sofia, Bulgaria (Poirel *et al.*, 2014; Savov *et al.*, 2012).

The Bacterial Challenge and Bad Practice

The discovery of antibiotics was a leap in modern medicine. However, the bacteria in particular have proven to be much more innovative and adaptive than scientists had imagined. Considering that the bacteria existed for a period of 3.8×10^9 years, while the antibiotics were used in the last 70 years, it is clear why there is no definitive success in bacterial infections' treatment (Hamilton-Miller, 1990). On the other hand, the bad practices and mismanagement have only exacerbated the situation. In 1998, in the US, it was estimated that there were 80 million prescriptions of antibiotics for human use - the equivalent of about 12 500 tons per one year (Yim, 2009). According to the American College of Physicians, 190 million doses of antibiotics are administered each day in the hospitals (www.acponline.org).

Among outpatients, more than 133 million courses of antibiotics are prescribed by doctors each year. It is estimated that 50 percent of the latter prescriptions are unnecessary since they are being prescribed for colds, coughs, and other viral infections. When animal and agricultural uses of antibiotics are added to human use, it is estimated that in the past 50 years, more than one million tons have been produced and disseminated (Yim, 2009). To combat the occurrence of resistant bacteria, pharmaceutical companies must constantly research, develop, and test new antimicrobials in order to maintain a pool of effective drugs on the market. Five years ago, there were approximately 150 antibiotics available to the public with new drugs appearing once every 8-10 years. This appears to be a substantial amount (Yim, 2009). However, these numbers are misleading because many of the targets of these drugs are similar. Since the drug development process is very expensive, pharmaceutical companies often concentrate on finding antimicrobials similar to the ones

already found, to reduce the risk of producing an unmarketable drug. This means that it is easy for a microorganism to develop resistance to a similar drug to which it already has resistance. Past and current strategies to combat resistance are not effective (Yim, 2009). In this sense, it can be concluded, that there is a crisis in the lack of new antibiotics to deal with the evolving and predictable problem of antibiotic resistance, and a critical need for agents active against multidrug-resistant Gram (-) bacilli (Top 10 Infectious disease publications in 2009) (<http://www.medscape.com>).

Perspectives, Cooperation Against Resistant Bacteria

The review of data from the European Union shows that 25 000 deaths per year were attributed to infections caused by antibiotic-resistant bacteria, of which 66% were Gram (-) bacilli. The total number of additional hospital days required for treatment of resistant bacteria is 2.5 million days per year at a cost of 1.5 billion euros (Top 10 Infectious disease publications in 2009) (<http://www.medscape.com>). Although this conclusion is not new, the document is extremely full of substantial data provided by the Antibiotic Availability Task Force from the Infectious Diseases Society of America (IDSA), which has catalogued much of these data over the past 5 years. The difference here is that the conclusion has particular meaning when it comes from the European equivalent of the CDC and the European equivalent of the US Food and Drug Administration (FDA) (Top 10 Infectious disease publications in 2009) (<http://www.medscape.com>). In this connection, at the EU-US Summit on November 3, 2009 in Washington, the president B. Obama, Jose Manuel Barroso, Fredric Reinfeldt, and Javier Solana were agreed to establish a transatlantic task force on urgent antimicrobial resistance issue (EU-US Summit agrees to form transatlantic task force on antimicrobial resistance.) (www.reactgroup.org).

The task force has to focus on appropriate therapeutic use of antimicrobial drugs in the medical and veterinary communities, prevention of both healthcare and community-associated drug resistant infections, and strategies for improving the pipeline of new antimicrobial drugs, which could be better addressed by intensified cooperation between the US and Europe. Following this, the IDSA Antibiotic Availability Task Force announced the necessity to achieve the development of ten new antibiotics within the next ten years (the 10 × '20 initiative),

meaning that the aim is to develop 10 novel drugs for Gram (-) bacteria by the year 2020 (EU-US Summit agrees to form transatlantic task force on antimicrobial resistance), (Top 10 Infectious disease publications in 2009) (www.reactgroup.org, <http://www.medscape.com>). However, these numbers are misleading because many of these drug targets are similar.

Since the drug development process is very expensive, pharmaceutical companies often concentrate on finding antimicrobials similar to the ones already found to reduce the risk of producing an unmarketable drug. This means that it is easy for a microorganism to develop resistance to a similar drug to which it already has resistance (Yim, 2009). Past and current strategies to combat resistance are not effective.

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Review**Viruses in Antarctic Habitats: Occurrence and Ecological Importance**Angel S. Galabov¹, Victoria Gesheva¹, Teodor Negoita²¹*The Stephan Angeloff Institute of Microbiology, Bulgarian Academy of Sciences, 1113 Sofia, Bulgaria*²*Romanian Polar Research Institute, Bucharest, Romania***Abstract**

The review describes the spreading of viruses in Antarctic ecosystems including terrestrial, marine, and freshwater habitats. The great attention is paid to virus infections in Antarctic animal and plant populations. Observation on viruses in Antarctic ecosystems might contribute for their diversity and systematics, as well as for viral ecology. Comparison of the detected viruses with the well-known viruses, using modern methods, reveals some structural differences in their nucleic acids and proteins. Several new virus species unknown in temperate geographic regions were described. Distribution of viruses in Antarctica may elucidate their migration, potential reservoirs, and hosts. Observations on viruses would explain the processes of virus infections, survival, pathogenicity, and medicine.

Key words: viruses, Antarctica, terrestrial and aquatic ecosystems, animal and plant hosts, bacteriophages

Резюме

Обзорът описва разпространението на вируси в екосистемите на Антарктида, включително земните, морските и пресноводните находища. Особено внимание е обърнато на вирусните инфекции в животинските и растителните популации в този континент. Наблюдението на вирусите в антарктическите екосистеми би могло да допринесе за проучване на тяхното разнообразие и систематика, както и за екологията им. Извършено е сравнение на изолираните в Антарктида вируси с познатите вируси чрез използване на съвременни методи, разкриващи някои структурни разлики в техните нуклеинови киселини и протеини. Описани са няколко нови вида вируси, непознати в географските региони с умерен климат. Разпространението на вирусите в Антарктида би могло да осветли тяхната миграция, потенциални резервоари и гостоприемниците им. Наблюденията върху вирусите в Антарктида би могло също така да обясни някои процеси във вирусните инфекции, тяхното преживяване, патогенност и медицинско значение.

Introduction

Viruses occupied an extremely important place in the life of our planet. They were proved as causative agents of more than 93% of infectious diseases. Moreover, they are etiologically connected with a substantial part of the neoplasms. It is noteworthy to stress the increasing role of emerging viral infections in the end of 20th and the beginning of the 21st century. As principal factors in the appearance of novel virus infections could be considered (i) viral adaptation and evolution: viruses more than all other microorganisms possess the capability to adapt to their hosts, to the environment, and to new ecological niches supplied by the man when he invades their territory; (ii) the increase and the movement of the global population (the substan-

tially increased transport communications); (iii) human activities; and (iv) the ecological factors and changes in the environment. The changes in the environment as a result of the human intervention lead to new ecosystems. During the last years we are witnesses of many examples of a global menace for the human population by diseases with origin in other species of the animal world - the so-called zoonoses. Their emergence is strongly dependent on both the climate changes and the environment changes caused by the human activities.

Tropical and subtropical areas of the Earth are the main objects of the research on the emerging viral infections, predominantly arboviral infections, as well as on another studies connected with the sources of emerging and re-emerging viral in-

fections on a global scale. A great number of investigations are directed to the clearing up of the factors contributing to the spread of viruses and the virus infections in the Earth areas with a temperate climate (mainly Europe and North America). In contrast with the latter, studies on viral progenies in the zones around poles - Arctica and Antarctica - are comparatively scarce. There is a reason to believe that viral progenies from these zones could be involved in the processes of the spreading of the viral infections around the globe.

The genome structure of viruses ensures their replication in different ecosystems, including terrestrial, marine, and freshwater habitats, and live in microbial, plant, and animal hosts. A review has already been published on the role of viruses in microbial communities in the Antarctica (Pearce and Wilson, 2007). However, a systematic view on the viruses in animal hosts in Antarctic area is not done. Besides, little data exist on viruses associated with plants, including mosses (Polischuk *et al.*, 2007), although viruses have been observed in *Stilborcarpa* (Skotnicki *et al.*, 2003).

All this shows a need for profound researches on viruses found in the polar areas, predominantly in Antarctica, their reproductional dynamics, survival, and persistence in different substrates and hosts. This is the main task of the present review.

Viruses in Animal Hosts

The possibility of the introduction of infectious agents to Antarctica animal life and its spread from Antarctica to other continents is discussed by the Antarctic Treaty. The migratory birds could be a potential vector for infectious agents.

Data on the occurrence of avian pathogen **infectious bursal disease virus** (IBDV) in Antarctic penguins near the centers of human activities indicated a possibility of virus introduction (Gardner *et al.*, 1997). Although IBDV is a pathogen of domestic chicken (*Gallus domesticus*), antibodies have been found in a variety of world bird species (Wilcox *et al.*, 1983). IBDV was detected in emperor (*Aptenodytes forsteri* Gray) and Adélie (*Pigoscels adeliae*) penguins.

Influenza A viruses and **paramyxoviruses** are widely distributed among wild birds in temperate regions which play a role in the spread of pathogenic paramyxoviruses and Newcastle disease virus (NDV). There is evidence that aquatic wild birds are the reservoir of all influenza viruses for avian and mammalian species, including humans (Webster *et al.*, 1992).

Paramyxoviruses have been found in Adélie penguins at Cape Bird and Casey, some 4 000 km away, in Antarctica (Morgan and Westbury, 1981, 1988; Austin and Webster, 1993). Viruses isolated from a cloacal swab taken from an adult penguin were classified as paramyxoviruses according to their morphology (Morgan and Westbury, 1981; Alexander *et al.*, 1989). On Macquarie Island - a sub-Antarctic island in the Southern Ocean - six paramyxoviruses, including a low pathogenic strain of NDV, were also isolated from cloacal swabs collected from 831 penguins of four species (Morgan *et al.*, 1981; Morgan, 1988). Samples of 100 penguins from King George Island were tested by real-time polymerase chain reaction (PCR), two of which have been positive for NDV (Thomazelli *et al.*, 2010). Antibodies to a **flavivirus** and an avian paramyxovirus, different from NDV, were detected in three of the four penguin species. The detection of an avian paramyxovirus from an apparently healthy Adélie penguin, and the presence of paramyxovirus and influenza virus antibodies in penguins and skuas, *Stercorarius skua maccormicki* (Saunders), demonstrated that these viruses may infect the avifauna of the Ross Sea Dependency (Austin and Webster, 1993). Sera from 1002 penguins of four species on Macquarie Island, a sub-Antarctic island in the Southern Ocean, were examined for antibody to NDV and other avian paramyxovirus, influenza A virus, alphavirus, and flavivirus. Sera from 6% of royal penguins sampled gave positive reactions to NDV, while to the other three species were negative. Six paramyxoviruses, different from NDV, were obtained from swabs taken from royal and king penguins at two widely separated sites on the island. Miller *et al.* (2010) isolated a paramyxovirus from rockhopper penguins (*Eudyptes chrysocome*) in Falkland Islands and suggested that this virus represented a new avian paramyxovirus (APMV) group - APMV10. Its viral hemagglutination (HA) activity was not inhibited by antisera against any of the nine defined APMV serotypes.

Penguin blood samples collected at Bird Island, a sub-Antarctic South Georgia Island, gave serologic indications that **influenza A virus** is present amongst these penguin species (Wallensten *et al.*, 2006). Baumeister *et al.* (2004) showed an infection of the birds, giant petrels, *Macronectes giganteas*, and penguins in Southern Shetland Islands with influenza A viruses of the subtypes A (H1), A (H3), A (H7), and A (H9). Recently, avian influenza virus A(H11N2) was isolated from Adélie penguins (<http://mbio.asm.org/content/5/3/e01098-14>).

full.pdf+htm). This virus is considered as a representative of evolutionary distinct lineage of avian influenza A virus (Hurt *et al.*, 2014).

Edison Durigon who coordinates a group of hunters of dangerous viruses in Sao Paulo, Brazil, that monitors birds and mammals from the Amazon to the subpolar islands said on August 23, 2009, that they have the first evidence that influenza is circulating among penguins in Antarctica. His new data, not yet submitted to the scientific community, indicate that the presence of influenza in this region is high, between 8-10% in a study population of one hundred animals. In other communities of birds from the icy continent, which have been analyzed, the occurrence of the same virus usually alternates around 1%. None of Antarctic penguins studied had symptoms of the disease.

The human population in Antarctica increases, because of the presence of the scientists and the growth of tourism in the region. Almost 40 000 tourists visited the continent only in the summer of 2008/2009, according to data from IAATO (International Association of Tour Operators in Antarctica). This is coupled with the fact that different **influenza virus** strains like to swap genes between each other, which sometimes leads to occurrence of a more aggressive virus. Thus, a potentially harmful scenario was riding possibly transforming the Antarctic in a nest of influenza virus strains, which could migrate to another regions. Evidently, the polar regions deserve an increased attention on the transmission of viruses (from who and to whom the viruses are passed), or what type of influenza virus is in circulation (seasonal human or avian H5N1). The penguins live along the international monitoring of the influenza viruses. According to Jansen Allen from the Durigon's research group the studies conducted so far do not allow us to say with certainty the side of migratory birds such as skuas and giant petrels - the analysis of their faeces were also positive for influenza - as well as of mammals such as elephant seals and sea lions (<http://www1.folha.uol.com.br/folha/c...6u613636.shtml>). Penguin blood samples collected at Bird Island, a sub-Antarctic South Georgia Island, gave serologic indications that influenza A viruses present amongst these penguin species (Wallensten *et al.*, 2006).

As concerns DNA containing viruses in Antarctica, the group of Alcami isolated in Antarctic lakes numerous unknown DNA viruses varying in size (Lopez-Bueno *et al.*, 2009). Later, a novel adenovirus species was isolated from Chinstrap penguins (*Pygoscelis antarctica*), designated Chinstrap

penguin adenovirus type 1 (CSPAdV-1) (Lee *et al.*, 2014). A novel papillomavirus among the Adélie penguins (*Pygoscelis adeliae*) in Ross Island was also found by an Australian research group (Varsani *et al.*, 2014). Antibodies to **poxvirus** were found in Antarctic Peninsula by Shearn-Boschler *et al.* (2008).

Have *et al.* (1991), and Heidejorgensen *et al.* (1992) detected evidence for prior infection by phocine **distemper virus** in Arctic and Antarctic seal populations, while Harder *et al.* (1991) found the antibodies against phocine **herpesvirus** in sera from Antarctic seals. Stenvers *et al.* (1992) reported that Weddell and crabeater seals in the Antarctica had high neutralizing titres to phocine, canine, and feline herpesviruses. In the same time, antibodies to canine distemper virus were detected in Antarctic seals by Bengtson *et al.* (1991). Harder *et al.* (1991) pointed that spreading of viruses may be done by nomadic movements of crabeater seals in a wide geographical region.

Rockhopper (*Eudyptes chrysocome*), royal (*Eudyptes schlegeli*), and king (*Aptenodytes patagonicus*) penguins living in Macquarie Island, a small sub-Antarctic island, are harbours for ticks (*Ixodes uride*). It was established that associated with penguin ticks carried four genera **arboviruses**: a flavivirus, an orbivirus, a phlebovirus, and a nairovirus (Morgan, 1988; Major *et al.*, 2009). The **flavivirus** was nearly identical to Gadgets Gully isolated 30 years ago, which showed the remarkable genetic stability of this virus. The authors named the detected **orbivirus** as Sandy Bay virus and considered that the nearest relative to the orbivirus is the Scottish Broadhaven virus, and it provided only the second available sequences from the Great Island orbivirus serogroup. For **phlebovirus** they propose the name Catch-me-cave virus and believe that it is distinct, but related to the previously isolated Precarious Point virus showing homology with the Finnish Uukuniemi virus. These viruses, isolated from penguins, provided the second and third available sequences for the Uukuniemi group of phleboviruses. The **nairovirus** named Finch Creek virus was shown to be related to the North American Tillamook virus, the Asian Hazara virus, and Nairobi sheep virus. Authors concluded that Macquarie Island penguins carried arboviruses from at least four of the seven arbovirus-containing genera, with related viruses often found in the Northern Hemisphere

Polar skuas, *Catharacta maccormicki* Saunders, living around Antarctic Davis Station, had no

ticks and the authors were not able to isolate any viruses (Miller *et al.*, 2008). It was established that skuas were seropositive to some avian viruses: 16.9% had antibodies to infectious bursal disease virus and 10.5% were seropositive for Newcastle disease. One percent of skuas had antibodies to avian influenza and there was no evidence of egg drop syndrome, but 27.8% had antibodies to flaviviruses. Perhaps, polar skuas encounter a variety of pathogens either in Antarctica or during their migration in the non-breeding season.

Viruses in seals. Tryland *et al.* (2005) isolated from skin lesion on the neck of a Weddell seal (*Leptonychotes weddellii*), in Queen Maud Land, Antarctica, proliferative papilloma-like structures. Electron microscopy revealed typical **parapoxvirus** particles.

New strains of murine cytomegavirus in wild mice were isolated by Booth *et al.* (1993) and arboviruses, including both new flavivirus and bunyavirus from *Ixodes uriae* detected at Macquarie Island (Doherty *et al.*, 1975; St George *et al.*, 1985). Linn *et al.* (2001) isolated a novel **alphavirus** from the louse *Lepidophthirus macrorhini*, collected from southern elephant seals (SES), *Mirounga leonina*, on Macquarie Island, Australia. The virus possessed classic alphavirus ultrastructure and appeared to be serologically different from any known Australasian alphaviruses. All Macquarie Island elephant seals tested had neutralizing antibodies against the virus. Sequence analysis demonstrated that the SES virus segregates with the Semliki Forest group of Australasian alphaviruses. Phylogenetic analysis of the known alphaviruses suggests that they might be grouped according to their enzootic vertebrate host class. The SES virus is an arbovirus of marine mammals and can be transmitted by lice.

Czaker (2002) found an unknown *Aggregata* sp. in the renal organ of an antarctic *Benthoctopus* sp., when it was inspected for the presence of dicymid mesozoans. Merozoites invaded the renal epithelium, while sporogony stages resided in the submucosal connective tissue. Individuals of all developmental stages were found to be infected with unknown virus-like particles. They are spherical in shape, non-enveloped, and measuring approximately 30 nm in diameter, and could be observed in the nuclei as well as within the cytoplasm. Virus-like particles had a feature to arrange in paracrystalline arrays. No pathogenic effect on the parasites was detected. Based on the host specificity, size, morphology, and histochemical analysis, which sug-

gested the putative viral genome as RNA, a relationship with **Totoviridae** was assumed.

Viruses in Plants

Skotnicki *et al.* (2003) described plant *Stilborcarpa* mosaic bacilliform badnavirus (SMBV) from Macquarie Island. Barley yellow draft virus-PAV was isolated in the sub-Antarctic Kerguelen Islands, two new luteovirus species been proved (Svanella-Dumas *et al.*, 2013). Plant samples of four moss genera - *Polytrichum*, *Plagiatecium*, *Sanionia*, and *Barbilophozia*, and one plant species, *Deschampsia antarctica* - were collected, and they were subjected to enzyme-linked immunosorbent assay of testing for the presence of common plant viruses (Polischuk *et al.*, 2007). Samples of *Barbilophozia* and *Polytrichum* mosses contained antigens of viruses from the genus *Tobamovirus*, tobacco mosaic virus, and cucumber green mottle mosaic virus, which normally parasitize. Viral agents mainly from order *Caudovirales* were found in soil samples (Polischuk *et al.*, 2007).

Viruses and Bacteriophages in Polar Aquatic Ecosystems

It was established that viruses are ecologically significant component of water saline and fresh environments (Hennes *et al.*, 1995; Weinbaur and Hofle, 1998; Wilhelm and Smith, 2000). Viruses that infect eukaryotic phytoplankton are an important member of aquatic ecosystems and they control a number of potential host species, however in lakes they remain largely unobserved yet. Although certain virus antibodies have been found, the presence of viruses is difficult to evaluate - only a small number of disease-causing viruses were isolated (Barbosa and Palacios, 2009).

Antarctic lakes are extreme ecosystems in which viruses may be important in controlling microbial community dynamics. Kepner *et al.* (1998) showed that water samples collected from four ice-covered Antarctic lakes contained high densities of extracellular viruses. Majority of these viruses were found to be morphologically similar to double-stranded DNA viruses that are known to infect algae and protozoa. The abundance of planktonic viruses indicates that viral lysis may be a major factor in the regulation of microbial populations in these extreme environments. Water samples from fresh-water Antarctic lakes on Signy Island (in the South Orkney Islands) contained virus-like particles (VLP), ubiquitous, morphologically diverse, and abundant, with high concentrations ranging

from $4.9 \times 10^6 \text{ ml}^{-1}$ to $3.1 \times 10^7 \text{ ml}^{-1}$. The hosts included bacteria, cyanobacteria, and eukaryotic algae. In addition, an unusually large virus morphotype was observed with a head diameter of $370 \times 330 \text{ nm}$ and a $1.3 \text{ }\mu\text{m}$ long tail (Wilson *et al.*, 2000). Lisle and Priscu (2004) showed that in McMurdo Dry Valleys of Antarctica lakes total bacterial abundances ranged from 3.8×10^4 to $2.58 \times 10^7 \text{ cells ml}^{-1}$ and VLP abundances ranged from 2.26×10^5 to $5.56 \times 10^7 \text{ VLP ml}^{-1}$. VLP abundances were significantly correlated ($p < 0.05$) with total bacterial abundances, bacterial productivity, chlorophyll *a*, and soluble reactive phosphorus, while lysogenic bacteria, determined by induction with mitomycin C, made up between 2.0% and 62.5% of the total population of bacteria. The contribution of viruses released from induced lysogens was $< 0.015\%$ of the total viral production rate. Carbohydrate and protein forming organic aggregates in lake water were abundant and were heavily colonized by bacteria and VLPs. VLP abundances in nine freshwater to saline lakes in the Vestfold Hills, Eastern Antarctica, were determined by Laybourn-Parry *et al.* (2001). In the ultra-oligotrophic to oligotrophic freshwater lakes VLP abundances ranged from 1.01 to $3.28 \times 10^6 \text{ ml}^{-1}$ in the top 6 m of the water column, while in the saline lakes the range was between 6.76 and $36.5 \times 10^6 \text{ ml}^{-1}$. The lowest value was detected in Ace Lake and the highest value in hypersaline Lake Williams.

Virus-to-bacteria ratios were lowest in the freshwater lakes and highest in the saline lakes. Divergent morphologies among VLP was observed, including phages with short (*Podoviridae*) and long tails, icosahedral viruses of up to 300 nm and star-like particles of about 80 nm diameter. In these ecosystems dominated by microbes, no correlation between VLP and bacterial numbers was found. There was a significant correlation between VLP abundances and dissolved organic carbon concentration. The data indicated that viruses attack bacteria and protozoan species. VLP numbers in the freshwater lakes were lower than values indicated for lower latitude systems. Those in the saline lakes were comparable with abundances found from other Antarctic lakes, and were higher than most values published for lower latitude lakes and many marine systems. S  wstr  m *et al.* (2008a, b) studied distribution of VLP in ten lakes in the Vestfold Hill, Antarctica, and detected lysogeny, determined with mitomycin C only in one of the lakes, indicating that viral replication was occurring predominately via the lytic cycle.

Principal component analysis and confirmatory correlation analysis of individual variables confirmed that high abundances of VLP occurred in lakes of high conductivity with high concentrations of soluble reactive phosphorus and dissolved organic carbon. In lakes, possessing high concentrations of chlorophyll *a*, intact bacteria, and rates of bacterial production, viruses-to-bacteria ratios were established. Authors concluded that viral abundance in the Antarctic lakes was determined by the trophic status of the lake and the resultant abundance of intact bacterial hosts. Viral abundance in two large ultraoligotrophic freshwater lakes (Lake Druzhby and Crooked Lake) in the Vestfold Hills, Eastern Antarctica, ranged from 0.16 to $1.56 \times 10^9 \text{ particles l}^{-1}$ and bacterial abundances ranged from 0.10 to $0.24 \times 10^9 \text{ cells l}^{-1}$, with the lowest bacterial abundances noted in the winter months (S  wstr  m *et al.*, 2007). Virus-to-bacteria ratios (VBR) were consistently low in both lakes ranging from 1.2 to 8.4 .

Lysogenic bacteria were determined by induction with mitomycin C. In Lake Druzhby and Crooked Lake, lysogenic bacteria made up between 18% and 73% of the total bacteria population. Bacterial production ranged from 8.2 to $304.9 \times 10^6 \text{ cells l}^{-1} \text{ day}^{-1}$ and lytic viral production ranged from 47.5 to $718.4 \times 10^6 \text{ virus-like particles l}^{-1} \text{ day}^{-1}$. In the winter months, there was a high contribution from viruses when $> 60\%$ of the carbon supplied to the dissolved organic carbon (DOC) pool (i.e., autochthonous sources), originated from viral lysis while during the summer $< 20\%$ originated from viral lysis.

Madan *et al.* (2005) investigated viral and microbial loop dynamics in three saline lakes: Highway Lake (salinity of 4‰), Pendant Lake (salinity of 19‰), and Ace Lake (salinity of 18‰) in order to assess the importance of viruses in extreme, microbially dominated systems. VLP showed no clear seasonal pattern, with high concentrations occurring in both winter and summer, ranging from $0.89 \times 10^7 \pm 0.038$ to $12.017 \times 10^7 \pm 1.28 \text{ ml}^{-1}$. VLP abundances was connected with lake productivity based on chlorophyll concentrations. Pendant Lake, the most productive of the three lakes, supported the highest bacterial biomass, while the virus-to-bacteria ratios were higher in Ace Lake (range 30.58 ± 3.98 to 80.037 ± 1.6). The heterotrophic nanoflagellates of Highway Lake (dominated by the marine choanoflagellate *Diaphanoeca grandis*) showed a positive correlation with VLP. Highest rates of lysogeny - up to 32% in Pendant Lake and 71% in Ace Lake - were detected in winter and

spring, while there was no or lower lysogeny in the summer (Laybourn-Parry *et al.*, 2007).

In winter, VLP persisted in cold, dark water. High VLP concentrations and high VBR indicated that viruses, most of which were bacteriophages, are a major habitant within the microbial communities in extreme saline lakes. Variability in abundance of VLP, VLP decay rates, and prokaryotic mortality due to viral infection were determined in three Antarctic areas: Bellingshausen Sea, Bransfield Strait, and Gerlache Strait (Guixa-Boixereu *et al.*, 2002). VLP abundance showed very small spatial variability in the three areas and decreased from the surface to the bottom. Low seasonal variability in VLP abundance was found when comparing each area separately. Prokaryotic mortality due to viral infection was always higher than the one due to bacterivores.

To comprehend the nature of virus-host interaction in the sea, Sullivan *et al.* (2005) sequenced and characterized DNA genomes of three marine phages. One of them is podovirus and the other two are myoviruses. The marine phages are similar to two terrestrial phages, T4 and T7, which infect *Escherichia coli*, but also carry genes that appear specially adapted to infecting photosynthetic bacteria *Prochlorococcus* in nutrient-poor oceans. All three cyanophages contain photosynthetic-related genes and, thus, the virus helps the host maintain photosynthesis during infection. Toparceanu and Negoita (2007) investigated water samples from Lasermann Hills. The latter samples contained algal virus-like particles (PBCV). Some of them were identified and sequenced: chlorovirus (PBCV-1), 331 kb, 375 genes, with host *Chlorella*; Ectocarpus siliculosus virus (ESV-1), 336 kb, 231 genes, with host *Ectocarpus siliculosus*; coccolithovirus (EHV-86), 407 kb, 472 genes, with host *Emiliania huxleyi*. In these viruses 14 ancient genes were detected. The scientists revealed also prasinovirus (MPV-1), with host *Micromonas pusilla*, primnesiovirus (CBV), with host *Chrysochromulina brevifilum*, and raphidovirus (HAV-1), with host *Heterosigma akashiwo*. Lopéz-Bueno *et al.* (2009) indicated that the genetic structure of an Antarctic lake viral community revealed unexpected genetic richness distributed across the highest number of viral families. Antarctic virus assemblage had a large proportion of sequences related to eukaryotic viruses, including phycodnaviruses and single-stranded DNA (ssDNA) viruses not previously identified in aquatic environments. The authors revealed that the transition from an ice-covered lake in spring to an openwater

lake in summer led to a change from ssDNA to a double-stranded DNA-virus-dominated assemblage, which is possibly a seasonal shift in host organisms.

Borriss *et al.* (2003) isolated three host-specific bacteriophages from Arctic sea ice and melt pond samples collected north-west of Svalbard (Arctic). They belonged to the tailed, double-stranded DNA phage families *Siphoviridae* and *Myoviridae*. Their bacterial hosts exhibited the greatest similarity to the species *Shewanella frigidimarina*, *Flavobacterium hibernianum*, and *Colwellia psychrerythraea*.

As an unusual discovery could be considered the isolation by R. Cavicchioli and coll. (Yau *et al.*, 2011; Pyper, 2011) of a virophage, a virus that attacks viruses, from an extremely salty Organic Lake in Eastern Antarctica, named Organic Lake Virophage (OLV). It was established that this virus kill phycodnaviruses (Zablocki *et al.*, 2014).

Impact and Importance of the Studies on Antarctic Viruses

Studies on the levels of virus-induced mortality in Antarctica manifested the greatest impact of viral infection occurred under oligotrophic conditions in alpine lakes (Murray and Eldridge, 1994; Laybourn-Parry *et al.*, 2001; S  wstr  m *et al.*, 2007). A higher virus-to-bacteria ratio has been showed for eutrophic waters (Bratbak *et al.*, 1990). Viruses in Antarctica influenced biochemical and ecological processes as nutrient cycling, bacterial and algal biodiversity, algal bloom control, and genetic transfer.

Antarctica provides a good source of new organisms and model virus-host associations. They are often trapped in glacial ice and subglacial lakes. Conditions in Antarctica create the selection pressures including temperature, nutrient limitation, desiccation, and non-ionizing radiation. Specific environmental stress on microbial population communities can be used in observations of global climatic changes. Infectivity of free viruses may be lost by solar radiation (Suttle and Shen, 1992; Wommack *et al.*, 1996). Viruses participate in the maintenance of species diversity and genetic exchange.

By processes of lysogeny and pseudolysogeny the phage population may be maintained. Viruses in Antarctic lakes play an important part in controlling microbial community dynamics. High abundances of VLP occurred in lakes with high concentrations of soluble reactive phosphorus and dissolved organic carbon. Sullivan *et al.* (2005)

showed that marine phages, living in lakes and controlling the microbial dynamics, possess genes that appear specially adapted to infecting photosynthetic bacteria *Prochlorococcus* and, thus, the virus favours the host photosynthesis during infection. López-Bueno *et al.* (2009) revealed that the viruses in lakes have a great flexibility of DNA expressing in case of transition of single-stranded DNA to double-stranded DNA-virus-assemblages in dependence of season - spring or summer.

Observations on viruses in Antarctica are important for their diversity and systematics. Comparison of detected viruses with well-known viruses using modern methods reveals some differences in cell structures: proteins and nucleic acids. All this allows determination of the new species unknown in temperate geographic regions. These investigations are contributions in virus systematic and microbial ecology. Distribution of viruses in Antarctica may elucidate their migration, potential reservoirs, and hosts. Observations on viruses would explain the processes of virus infection, survival, pathogenicity, and medicine.

Investigations in the Antarctic area contribute in another highly fundamental scientific domain. Researchers on one of the subglacial lakes, Lake Ellsworth, situated in West Antarctic Ace Shelf, believe some of these isolated lakes could be home to microorganisms unknown to science, potentially holding secrets to the origine and limits of life on Earth. This knowledge could in turn hold clues about the possible conditions for extraterrestrial life, in locations such as the bodies of water through to exist beneath the surfaces of ice moons of Saturn and Jupiter (Pearce, 2012).

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Review**RNAi - Strategy to Control Viral Infections in Eukaryotic Organisms**Nikolay M. Petrov^{1,2*}, Angel S. Galabov²¹*Institute of Soil Science, Agrotechnologies and Plant Protection "Nikola Pushkarov", Kostinbrod*²*The Stephan Angeloff Institute of Microbiology, Bulgarian Academy of Sciences***Abstract**

Short double-stranded RNA molecules efficiently inhibit gene expression in eukaryotic cells. One class of these short molecules are the small interfering RNAs (siRNAs), which after introduction into cells elicit efficient induction of post-transcriptional gene silencing known as RNA interference (RNAi). They are incorporated into a multimeric protein complex named RNA-induced silencing complex (RISC). While one of the two RNA strands is discarded, the antisense strand guides RISC to the complementary target RNA and induces its endonucleolytic cleavage. RNA silencing by post-transcriptional gene silencing (PTGS) is a mechanism of gene regulation in eukaryotes. It is similar to classical humoral immunity, which protects eukaryotes against viruses and transposons. A major problem for the long term inhibition of viruses is the emergence of escape mutants. This limitation, which is well-known for conventional antiviral therapy with low molecular weight drugs, is applicable to RNAi approaches as well. Virus escape as a consequence of the accumulation of point mutations in or close to the siRNA target site has been observed for various types of viruses. To counter this problem a novel technology of production of dsRNAs is used, targeted to essential gene regions for viral replication. The technology is based on the replication complex of the bacteriophage phi 6. A pool of siRNAs is produced in this way, targeted to specific gene region, which overlaps and silences the target, whether a mutation or recombination appear.

Key words: RNAi, siRNAs, viruses**Резюме**

Късите дВРНКи ефективно инхибират генната експресия в еукариотните клетки. Един от класовете на тези къси молекули са малките интерфериращи РНКи (миРНКи), които след въвеждане в клетката предизвикват ефективно индуциране на пост-транскрипционно генно мълчание известно като РНК интерференция (RNAi). Те се включват в мултимерен протеинов комплекс наречен комплекс за индуциране на РНК мълчание (RISC). Сенси веригата на миРНК се отстранява, докато антисенси веригата насочва RISC комплекса към комплементарна РНК прицелна секвенция и по този начин индуцира ендонуклеотично срязване на прицелната секвенция. РНК заглушаването чрез пост-транскрипционно генно мълчание (PTGS) е механизъм на генна регулация при еукариотните организми. Процеса е подобен на класическия хуморален имунен отговор, който предпазва еукариотните организми от нашествието на вируси и транспозони. Основният проблем при продължителното инхибиране на вирусите е възникването на резистентни мутанти. Това ограничение, което е добре известно при традиционната антивирусна терапия с нискомолекулни съединения важи също така и за РНК интерференцията. Вирусната резистентност като следствие от натрупването на точкови мутации в прицелната секвенция или в близост до нея е наблюдавана при различни вируси. За разрешаването на проблема е използвана нова технология за получаване на специфични дВРНКи, комплементарни на основни за вирусната репликация генетични региони. Технологията се базира на репликационния комплекс на бактериофага phi 6. По този начин се получава пул от миРНКи, насочени към специфичен генетичен регион, които припокриват и заглушават прицелната секвенция, без значение дали се е появила мутация или рекомбинация в нея.

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History of Gene Silencing

RNA gene silencing is a mechanism of gene regulation, that limits the transcript level by either suppressing transcription [transcriptional gene silencing (TGS)] or by activating a sequence-specific RNA degradation process [post transcriptional gene silencing (PTGS)]/RNA interference (RNAi). The evolutionary functions of RNAi and its related processes are designed for the protection of genome against invading mobile elements like viruses and transposons as well as for orchestrated functioning of developmental programs in eukaryotes. Prior to the discovery of RNAi, scientists applied various methods such as insertion of T-DNA elements and transposons, treatment with mutagens or irradiation and antisense RNA suppression to generate loss-of-function mutations. Antisense RNA technology was discovered in plants, when the inhibition of nopaline synthase gene was observed in tobacco cells, due to the expression of its corresponding antisense RNA (Rothstein *et al.*, 1987).

RNA silencing was first observed in plants, where attempts to overexpress endogenous genes by introducing transgenic copies of the endogenous gene, instead, resulted in blocked expression of both. It happened when scientists were trying to increase the purple pigmentation of petunia petals by sense overexpression of chalcone synthase, a gene related to anthocyanine pathway (Napoli *et al.*, 1990; Van der Krol *et al.*, 1990). The Ambrose Laboratory reported the first case of micro RNA (miRNA) in an attempt to silence heterochronic gene *lin-4* of *C. elegans* (Lee *et al.*, 1993), and the Fire and Mello Laboratories described the gene silencing effect of double-stranded RNA (dsRNA) in *C. elegans* by injecting dsRNA that corresponds to *unc-22*, responsible for body morphology. They took the antisense silencing approach a step further in *C. elegans* with simultaneous introduction of both the sense and antisense strands of the targeted mRNA, resulting in a tenfold higher potency in silencing of the targeted mRNA (Fire *et al.*, 1998).

In animals, RNA silencing was first reported when Guo and Kemphues (1995) injected RNA to block *par-1* mRNA expression in *C. elegans*, but found that the *par-1* mRNA itself also repressed *par-1*. Their paradoxical observations subsequently named as RNAi - inspired the experiment of Fire *et al.* (1998), who demonstrated that dsRNA was the trigger of gene silencing.

Three phenotypically different but similar by mechanism forms of gene silencing, co-suppression or PTGS in plants (Jorgensen, 2003), quelling in

fungi (Cogoni *et al.*, 1996) and RNAi in the animal kingdom (Fire *et al.*, 1998) have been described. More recently, miRNA formation (Pasquinelli, 2002; Bartel, 2004), promoter methylation (Matzke *et al.*, 2001; Wesley *et al.*, 2001), heterochromatinization (Schramke and Allshire, 2003), etc., have been revealed as other facets of naturally occurring RNAi processes in eukaryotic cells.

Different Classes of RNAs that Induce Gene Silencing

Ribosomal RNA (rRNA) is the most abundant type of RNAs inside the cell followed by transfer RNAs (tRNAs) and messenger RNAs (mRNAs). There are another four basic classes of RNAs, which take active parts in RNA silencing. These are hairpin RNAs (hpRNAs), double stranded RNAs (dsRNA), small interfering RNAs (siRNAs) and micro RNAs (miRNAs).

Double stranded RNA (dsRNA) is formed by the complementary base pairing of two single-stranded fragments of RNA and takes an active part in RNA silencing pathway (Agrawal *et al.*, 2003). The dsRNA found naturally in the cell and is derived generally from the replacement of transposons (Schramke and Allshire, 2003) or virus induction.

Small interfering RNA (siRNA), also known as short interfering RNA or silencing RNA, is a type of 20 to 25 nucleotide-long double-stranded RNA molecules with a 3' two nucleotide overhang, that take a variety of parts in the cell. This is formed from the long dsRNA by the cutting activity of a special enzyme called Dicer. The short interfering RNA is involved mainly in RNA interfering pathway, where it is involved in disrupting the function of a gene by interfering with the RNA expressed by that gene. siRNA was first discovered as a part of post-transcriptional gene silencing (PTGS) in plants by the group of David Baulcombe in Norwich, England (Hamilton and Baulcombe, 1999). Not much later in 2001, synthetic siRNAs have been reported to be involved in RNAi initiation in human cell line (Elbashir *et al.*, 2001). This discovery has been revolutionizing the field of gene-function analysis. Short interfering RNAs are believed to have an important role in viral resistance and in preventing transposons transposition (Lippman *et al.*, 2003).

Hairpin RNA (hpRNA) is another form of dsRNA. It is deduced from a long piece of single stranded RNA containing inverted repeat and connected by a hairpin (Wesley *et al.*, 2001). This long

piece of single stranded RNA is produced by a vector introduced in the cell. A constitutive promoter, U6 in animal cells and cauliflower mosaic virus 35S promoter (CaMV35S) in plants, is used to ensure the continuous expression of hpRNA in cell. Hairpin RNA is transcribed by RNA polymerase III in animal cell and by RNA polymerase II in plants miRNAs. These are small non-coding single stranded molecules of about 21-23 nucleotides in length, that negatively regulate gene expression, which were first discovered in the nematode *C. elegans*, while screening the genes that control developmental timing (Lee *et al.*, 1993), and hundreds of miRNAs have been identified in plants and animals, including the hundreds unique miRNA from *Arabidopsis* alone (Lee and Ambros, 2001; Llave *et al.*, 2002; Reinhart *et al.*, 2002; Millar and Waterhouse, 2005). Micro RNAs are formed from the precursor single-stranded RNA transcripts that have the ability to fold back onto themselves to produce imperfectly double-stranded stem loop precursor structures. The main function of miRNA is gene regulation (Grosshans and Slack, 2002).

Piwi-interacting RNA (**piRNA**) represents the largest class of small non-coding RNA molecules expressed in animal cells, deriving from a large variety of sources, including repetitive DNA and transposons (Klattenhoff *et al.*, 2008). piRNAs appear to act both at post-transcriptional and chromatin levels. They are distinct from miRNA due to at least an increase in terms of size and complexity. Repeat associated small interfering RNA (**ra-siRNAs**) are considered to be a subspecies of piRNA (Gunawardane *et al.*, 2007).

Mechanism of Gene Silencing

Mechanism of PTGS is a two-step model for RNAi. The first step, referred to as the RNAi initiating step, involves binding of the RNA nucleases (Dicers) to a large dsRNA and its cleavage into discrete ~21 to ~25nt RNA fragments (siRNAs). In the second step, these siRNAs join a multinuclease complex (RISC), which degrades the homologous single stranded mRNAs. The first class of RNA taking an active role in RNAi is dsRNA, which is formed by complementary base pairing of two single-stranded fragments of RNA (Agrawal *et al.*, 2003). Long dsRNAs generally derived from such events as transposition of transposable elements (Schramke and Allshire, 2004) or virus induction (Rovere *et al.*, 2002), with which the PTGS process is initiated. The dsRNA alone cannot degrade mRNA, but requires the assistance of two enzymes

- namely Dicer and RISC. Dicer, which was first discovered by Bernstein *et al.* (2001) in *Drosophila*, is a complex enzyme belonging to the RNase III family. It has four different domains with different functions. They are: (a) N-terminal helicase, (b) dual RNase III motifs, (c) C-terminal dsRNA binding domain, (d) PAZ (Piwi/Argonaute/Zwille) domain (Kuznetsov, 2003; Arenz and Schepers, 2003). The PAZ domain is believed to physically interact with the corresponding PAZ domain of the RISC complex. The dual RNase III motifs perform the actual cleavage of the dsRNA, hence the characteristic 5' phosphate and 3' hydroxyl residues on the resulting siRNAs. ARGONAUTE1 (AGO1) mediates the cleavage of miRNA-targeted mRNAs and maintenance of chromatin structure (Vaucheret, 2006). Experiments involving human Dicer showed that the cleavage mechanism of the enzyme is ATP independent (Kuznetsov, 2003). The helicase domain is also believed to take part in the process. The Dicer protein functions in two different pathways in silencing a gene by recognizing distinct types of precursor dsRNA. In the first pathway, Dicer cleaves long and perfect dsRNA structures that originated mainly from the protein-coding region to generate double stranded siRNAs, which guide the subsequent endonucleolytic cleavage of homologous RNAs with perfect base pairing interaction (Elbashir *et al.*, 2001). In the second pathway, Dicer can dice imperfect RNA duplexes predominantly derived from the regions between protein coding genes into short RNAs, which are subsequently recruited into a micro RNA ribonucleoprotein complex (miRNP) to further regulate translational inhibition or other PTGS effects (Hutvagner *et al.*, 2001; Voinnet, 2002). Accordingly, Dicer processes precursors dsRNAs to generate both siRNAs and miRNAs. Four types of Dicers involved in small interfering RNA biogenesis have been reported in *Arabidopsis* (Xie *et al.*, 2004 and 2005), they are DCL1 (miRNA), DCL2 (viral RNA), DCL-3 (endogenous siRNA), and DCL-4 (exogenous siRNA). RISC is the component of the RNAi machinery that uses siRNAs to track down and degrade the target mRNAs. First discovered in *Drosophila* by Hammond *et al.* (2000), RISC consists of both protein and RNA. The protein component of the complex has ribonuclease activity with the ability to cleave RNA. In addition to ribonuclease activity, RISC also contains a PAZ domain involved in regulation of RNA interference (RNAi). Additional RISC components include two RNA binding proteins, intronic and dFMR protein (Arenz and Schepers, 2003).

The DCR-2/R2D2 complex also facilitates incorporation of siRNA into the RISC complex (Liu *et al.*, 2006). RISC utilizes the siRNA and search for the complementary target mRNA. The sequence and structure of a siRNA determines which of its two strands is to participate in the RNA silencing pathway. Each siRNA dissociates from the Dicer active site soon after it is produced, its thermodynamics evaluated by the RNA silencing mechanisms and then, one strand is selectively loaded onto RISC and the other is destroyed. The degradation process is initiated once the successful location and cleavage of the complementary mRNA occurs by the siRNA-RISC complex. In case of translational repression pathway, small RNAs direct RISC bind to target mRNA and repress its translation process, rather than cleavage. Animal miRNAs typically, but not always, mediate translational repression rather than cleavage. Translational repression occurs at some stage after translational initiation, because the distribution of ribosomes along the length of the repressed mRNA undergoing active translation (Vauchert, 2006). Thus, these RNAs are not a single class of ~25nt, but instead are two distinct species with 21-22nt and 25nt in size. The 21-22nt siRNA represents the siRNA that guides the RISC ribonuclease to the target of PTGS (Elbashir *et al.*, 2001; Aigner, 2006). However, it is unlikely that the two classes of siRNA have the same function because they accumulate differently in locally and systematically silenced tissue. Lipardi *et al.*, (2001) found that the 3' hydroxyl group is required in order to direct RNAi *in vitro*. While, Dicer may incorporate siRNAs into RISC following their synthesis, they do not require the event to occur *in vivo*. RNA silencing pathways can be divided into those that require RNA dependent RNA polymerase (RdRPs) and those that do not. In *C. elegans* and *N. crassa*, RdRP is required for silencing (Sijen and Kooter, 2000). Two models have been proposed to explain the role of RdRPs. First, RdRPs might use primary siRNAs to prime the synthesis of dsRNA using the target mRNA itself as a template. The mechanism of in-trans silencing is most probably based on the presence of siRNAs that corresponds to the region of homology between silencing inducer and target RNA (Depicker and Montagu, 2003). Several studies on plants indicated that silencing could also spread to regions downstream of the target (5'-3' spreading; Braunstein *et al.*, 2002; Vaistij *et al.*, 2002). A remarkable feature of PTGS is that it is non-cell autonomous, i.e. it can be induced in tissue actively expressing a transgene and move as a

mobile transmissible signal originating from tissue where the same transgene is silenced (Jorgensen *et al.*, 1998; Fagard and Vaucheret, 2000). The signal appears to be a sequence that is specific and move unidirectional from source to sink tissues (Voinnet and Baulcombe, 1997; Sonoda and Nishiguchi, 2000). The spread of silencing signal depends on the transcription of the target RNA (Baulcombe, 2002).

Dicers

Naturally occurring small RNAs can be: (1) endogenous siRNA; (2) miRNAs involved in gene regulation; (3) transposon-derived small RNAs; (4) virus-derived siRNAs. All siRNAs are the products of degradation of long dsRNA by RNase III-like enzyme family, first discovered in *Drosophila* (Bernstein *et al.*, 2001). This enzyme was named Dicer in animals or Dicer-like (DCL) in plants. Dicers are multifunctional proteins and contain one or more dsRNA binding domains. The number of Dicers may vary in different organisms. Many animals encode only a single Dicer, *Drosophila* encodes two (Lee *et al.*, 2004), and four DCL homologues (DCL1, DCL2, DCL3 and DCL4) have been identified in *Arabidopsis thaliana* that function differentially in siRNA and miRNA biogenesis (Schauer *et al.*, 2002). Other organisms (like *C. elegans* and humans) used only a single DCL protein to process both categories of silencing initiation. DCL1 is mainly responsible for the processing of miRNAs (Herr *et al.*, 2005). Some other factors, such as HEN1 and HYL1 (a dsRNA binding proteins) also help the DCL1 in the generation of miRNAs (Vazquez *et al.*, 2004; Xie *et al.*, 2004). HEN1 is also involved in some other functions like, natural virus resistance and transgene silencing (Boutet *et al.*, 2003). DCL2 was found to be involved in the production of viral-derived 22-nucleotide siRNAs and antiviral defence (Gascioli *et al.*, 2005). This viral-derived siRNA production also required two RdRps (RdRp1 and RdRp6) depending on the kind of virus which infect the plant (Muangsan *et al.*, 2004; Xie *et al.*, 2004). DCL3 and RdRp2 play a role in the generation of longer class of endogenous siRNAs (24 nt) and RNA-dependent DNA methylation (Xie *et al.*, 2004). These endogenous siRNAs are involved in the initiation or maintenance of a heterochromatic state (Matzke *et al.*, 2004). DCL4 is involved in RNA silencing in plants and seems to produce 21-nucleotide siRNA component of the cell-to-cell silencing signal (Dunoyer *et al.*, 2005).

DCLs proteins have an interchangeable and overlapping role in both siRNA and miRNA pathways (Deleris *et al.*, 2006).

RISC Complex

RNA-induced silencing complex (RISC) is a multi-protein complex involved in different catalytic functions in the process of RNA silencing as mRNA cleavage and translational inhibition. The sequence specificity is provided by the small RNA molecules to RISC. Another proteins AGO have been found to be part with RISC in all organisms. The AGO proteins are essential for the slicing activity of mRNA. Ten members of the AGO family have been identified. AGO1 expression is regulated by a miRNA (miR168) indicating that the AGO1 protein regulates its own expression in a negative feedback loop (Vaucheret *et al.*, 2004). AGO1 involved in the slicing activity and process miRNA and certain classes of endogenous siRNAs but not the viral siRNAs (Baumberger and Baulcombe 2005). The role of AGO4 is in the production of the long siRNA of about 24 bp. Its involvement in long siRNA mediated chromatin alteration (histone methylation) also has been reported (Zilberman *et al.*, 2003). AGO2 is a part of RISC and it is essential for siRNA-directed RNA silencing, as reported in *Drosophila*. AGO2 has no role in the processing of miRNA, but the role of AGO1 was indicated (Okamura *et al.*, 2004). Most of the AGO proteins are involved in different parts of RNA silencing (Baulcombe, 2004).

Development of PTGS Therapeutics

The target mRNA is enzymatically cleaved from any organism with PTGS, leading to decreased levels of gene expression of the targeted gene and the corresponding protein. The specificity of this mRNA silencing is controlled very precisely at the nucleotide level. RNAi (PTGS) is a fundamental cellular mechanism that can also be used to rapidly develop novel drugs against any disease target. RNAi was considered a novel option to treat viral infections. The reduction in expression of pathological proteins through RNAi is applicable to all classes of molecular targets, including those that have been traditionally difficult to target with either small molecules or proteins including monoclonal antibodies. RNAi therapeutics are under clinical investigation for age-related macular degeneration (AMD) and respiratory syncytial virus (RSV) infection, with numerous other drug candidates.

Application of PTGS

Until the end of 1980s, only modifications of DNA or protein that lead to transcriptional repression or activation were classified as epigenetic drugs. During the 1990s, a number of gene-silencing phenomena at the posttranscriptional level were discovered in plants, fungi, and animals, introducing the concept of PTGS or RNA silencing (Baulcombe, 2000; Matzke *et al.*, 2001). The application of dsRNA technology for large-scale investigation of gene function can be facilitated by making the critical experimental procedures fast and efficient (Wesley *et al.*, 2001; Brummell *et al.*, 2003). RNA silencing is an adaptive immune response that restricts accumulation or spread of viruses (Morris and Rossi, 2006). The transfection of mammalian cells with exogenous siRNAs has rapidly been adopted as a technology for targeted gene silencing (Elbashir *et al.*, 2001). Transgene intended to generate only sense or antisense RNA also silence gene expression, especially in plants (Jorgensen, 2003; Baulcombe, 2004).

A vector construct containing an inverted repeat of the 3' UTR of the nopaline synthase gene from *Agrobacterium* has been developed to provide dsRNA region at the 3' end of the transcript (Brummell *et al.*, 2003). This method, which they termed as silencing by heterologous 3' UTRs (SHUTR), has been shown to operate effectively in *Arabidopsis* and *Lycopersicon*. Heterologous silencing was first reported by Hamilton *et al.* (2002), who used an inverted repeat of a 79bp fragment of the 5' UTR of tomato ACO1 transcript. Previous studies of transgene-induced PTGS in plants have suggested that gene silencing is initiated in the 3' region of the target gene (Sijen *et al.*, 2001). PTGS was induced in tobacco plants (Petrov and Stoyanova, 2011; Petrov, 2012) and potato plants cv. Agria by specific siRNAs for HC-Pro region of Potato virus Y (PVY) strain NTN, which effectively blocked the viral replication (Petrov *et al.*, 2015a). PTGS was induced in potato plants cv. Arinda also by specific dsRNAs and siRNAs for HC-Pro region of PVY, which effectively reduced systemic spread of the virus. Reduction of the expression of the HC-Pro gene of PVY^N in newly grown leaves of potato plants, and, hence, the viral replication in all inoculated plants with the virus was established. The old treated and PVY inoculated leaves of the potato plants remain infected and later defoliate. All new leaves of potato plants cv. Arinda (not treated) grown after treatment with dsRNAs and siRNAs,

and PVY inoculation remain virus-free (Petrov *et al.*, 2015b).

Successful RNAi applications have been reported for most classes of medically relevant viruses including HIV-1, HBV, HCV, RSV, SARS-coronavirus, influenza virus, and poliovirus (Haasnoot *et al.*, 2007, Mescalchin *et al.*, 2011; Leonard and Schaffer, 2005). Some of these approaches have already reached the stage of clinical testing. While RNAi-mediated therapies against HIV-1 and HBV are based on shRNA expression systems (Haussecker, 2008), the most advanced clinical phase II trial make use of chemically synthesized siRNAs against RSV, which are administered by a nasal spray (DeVincenzo *et al.*, 2010).

PTGS against HIV infections

HIV was the first infectious agent targeted by PTGS, because its life cycle and gene expression is well understood. Synthetic siRNAs and expressed shRNAs have been used to target all of the HIV encoded RNAs in cell lines (Coburn and Cullen, 2002). Despite the early successes of RNAi-mediated inhibition of HIV-encoded RNAs in cell lines, targeting the virus directly represents a substantial challenge for clinical applications because of the high viral mutation rate. Therefore, avoiding this problem is based on targeting cellular transcripts that encode functions required for HIV-1 entry and replication such as cellular cofactors - NF kappa beta, the HIV receptor CD4, and the co-receptors CCR5 and CXCR4 have all been down-regulated with the result of blocking viral replication or entry (Anderson and Akkina, 2005; Cordelier, *et al.*, 2003; Surabhi and Gaynor, 2002).

PTGS against CVB3

PTGS has been evaluated as novel strategy to inhibit CVB3 (Schubert *et al.*, 2005; Merl *et al.*, 2005; Ahn *et al.*, 2005; Yuan *et al.*, 2005). In order to investigate the mechanism of RNAi-mediated inhibition of CVB3 in more detail, siRNAs were intentionally designed to target the viral plus strand, the minus strand, or both (Schubert *et al.*, 2007).

Gene-Silencing Strategies to Prevent Viral Escape

A major problem for the long-term inhibition of viruses is the emergence of escape mutants. This limitation, which is well-known for conventional antiviral therapy with low molecular weight drugs, is applicable to RNAi approaches as well. Virus escape as a consequence of the accumulation

of point mutations in or close to the siRNA target site has been observed for various types of viruses, including poliovirus (Gitlin *et al.*, 2005) and HIV (von Eije *et al.*, 2008). Three counter-strategies have been followed to counter the problem of viral escape: (1) targeting of conserved regions of the virus genome; (2) combination of efficient antiviral siRNAs; and (3) silencing of host factors that are essential for the viral life cycle. Mutations in highly conserved regions of the virus genome are likely to cause a loss of virulence. Comparison of silencing by siRNAs against different genomic regions of CVB3 revealed that targeting of non-structural protein coding regions is superior to selecting structural protein coding regions, since enzymes often lose activity when mutations occur (Merl and Wessely, 2007). Several groups have initially directed siRNA against the 5' untranslated region (5'UTR) of the CVB3 genome, which harbors the internal ribosome entry site (IRES). Interestingly, none of the tested siRNAs exerted significant antiviral activity (Merl and Wessely, 2007; Kim *et al.*, 2007). A possible explanation for this unexpected finding is that tight structures such as the IRES are detrimental to siRNA-mediated gene silencing (Schubert *et al.*, 2005). Only after laborious screening for accessible sites of complementary oligonucleotides in the 5' UTR region could ensure siRNAs to target efficiently the IRES and inhibit CVB3 (Dutkiewicz *et al.*, 2008).

The antiviral activity of the siRNA was improved further by its partial modification with locked nucleic acids (LNA), which have a high affinity towards complementary RNAs. Another highly conserved region of the CVB3 genome is the *cis*-acting replication element (CRE) located in the 2C protein coding region. A siRNA directed against this region conferred sustained protection against CVB3 and prevented the emergence of viable escape mutants (Lee *et al.*, 2007). Since the CRE sequence is identical in other enteroviruses such as echoviruses 6 and 7, and A-type as well as other B-type coxsackieviruses, the siRNA has a universal and persistent anti-enteroviral activity.

A widely employed strategy to minimize viral escape in conventional virus therapy is to combine various agents with antiviral activity. For HIV, this approach is known as highly active anti-retroviral therapy (HAART) or combined anti-retroviral therapy. The adaptation of this idea to RNAi is the combination of two or more active siRNAs or shRNAs. Combination of two shRNA expression cassettes in one vector was found to maintain silencing activity

against mutated target RNAs of CVB3 in a reporter system, since the second shRNA can compensate for the loss of silencing activity of the shRNA directed against the mutated target site (Schubert *et al.*, 2005). A systematic investigation of viral escape in cell culture revealed that cocktails of three siRNAs targeting distinct sites of the virus genome could maintain therapeutic efficacy, while virus inhibition with dual- or single-molecule-based RNAi was abrogated by viral escape (Merl and Wessely, 2007). In the long run, however, resistant mutants are likely to develop even against combinations of three or more site-specific siRNAs. Therefore, using a pool of siRNAs covering 3.5 kb of CVB3 genomic sequence was developed (Nygardas *et al.*, 2009). The pool was generated by synthesizing a long double-stranded RNA covering the region encoding most of the non-structural proteins of CVB3, which was subsequently cleaved into siRNAs by a recombinant Dicer. The pool of siRNAs was found to be significantly more effective than single-site siRNAs. Although this strategy can be expected to prevent viral escape, its therapeutic application is questionable since the antiviral agent consists of a heterogeneous mixture of hundreds of different siRNA molecules. It is currently unclear whether the pool of siRNAs will induce more severe off-target effects than single siRNAs, since it consists of numerous sequences, each of them being able to potentially regulate non-target RNAs. The third strategy for sustained inhibition of viral spread is silencing of genes of the host cells that are required by the virus to enter cells and replicate. The advantage of this approach is that viruses have a limited capacity to adapt to host cell changes. CVB3 initially attaches to the decay-accelerating factor (DAF), which serves as a co-receptor, prior to the virus entering the host cells via CAR. Whereas, CAR is essential for cardiac CVB3 infection (Shi *et al.*, 2009), most CVB2, CVB4, CVB6, as well as some strains of CVB1, CVB3, and CVB5 do not bind DAF (Freimuth *et al.*, 2008). Silencing of CAR was found to prevent infection of the treated cells by CVB3 (Werk *et al.*, 2005; Fechner *et al.*, 2007). CAR is located in the tight junctions of epithelial cells (Freimuth *et al.*, 2008; Coyne and Bergelson, 2005) and its constitutive gene knockout was found to result in an embryonic lethal condition associated with cardiac defects (Asher *et al.*, 2005). Animals with a conditional knockout of CAR at a later time point of embryonic development (E11) survived to adulthood and did not have evident cardiac abnormalities (Chen *et al.*, 2006). Very detailed investiga-

tions of heart function, however, revealed a block of atrio-ventricular conduction developed in the absence of CAR in the adult mouse heart, which may lead to arrhythmia (Lisewski *et al.*, 2008; Lim *et al.*, 2008). Conditional knockout mice exhibited a complete atrio-ventricular block and various phenotypes in other organs as well (Pazirandeh *et al.*, 2011). It should, however, be noted that no direct conclusions can be drawn from knockout experiments for RNAi applications, since knockout animals completely lack CAR, while RNAi only results in a partial knockdown of target gene expression, possibly leaving enough for preventing these defects from occurring. Since CAR might have essential cellular functions, other host factors should be considered as targets for inhibitors that block CVB3 indirectly (Coyne *et al.*, 2011). Conserved regions from VP1 of the coxsackievirus genome was identified and used as targets for RNAi (Petrov *et al.*, 2011). dsRNAs specific for conserved part of VP1 of coxsackievirus B1 (CVB1) and CVB3 reduced virus titer from 4 to 3 Lg CCID₅₀ *in vitro* in HEp-2 cells (Petrov and Galabov, 2012a). siRNAs from the same gene region reduced CVB1 and CVB3 virus titer from 5 to 3 *in vitro* in HEp-2 cells (Petrov and Galabov, 2012b).

Application of PTGS against CVB3 in Animal Models

A siRNA targeting the 2A protease encoding genomic region was found to lead to significant reduction of viral tissue titers, attenuate tissue damage, and prolong survival in highly susceptible type I interferon receptor-knockout mice (Merl *et al.*, 2005). The siRNAs were applied by hydrodynamic transfection. This method involves high pressure injection of the nucleic acid into the tail vein, which does not lend itself to a therapeutic setting for humans. It was therefore necessary to develop alternative application routes for efficient RNAi-mediated inhibition of CVB3. Since delivery of chemically synthesized siRNAs across the endothelial barrier to cardiomyocytes is inefficient, even in the presence of transfection agents, transfer of shRNA expression cassettes by viral vectors appears to be the method of choice. Kim *et al.* used a lentiviral vector to deliver the expression cassette for the above mentioned shRNA against the highly conserved CRE region (Kim *et al.*, 2008). Mice were injected intraperitoneally with the lentiviral vector and were subsequently challenged with CVB3. Treated animals had significant reductions in viral titers, viral myocarditis, and pro-inflammatory cytokines, and,

most importantly, the survival rate was improved from 20% to 50% at Day 10 after infection (Fechner *et al.*, 2008). The *in vitro* studies demonstrate that RNAi is an efficient approach to inhibit CVB3 and subsequent *in vivo* studies confirmed viral vectors to be suitable vehicles for the delivery of shRNA expression cassettes to the heart. Thus, the technology has the potential to develop into a therapeutic option to treat humans with virus-induced myocarditis (Schonhofer-Merl and Wessely, 2010).

Conclusion

The implications of PTGS, once viewed as an impediment to genetic engineering of plants, may instead be of fundamental importance both for controlling gene expression of pathogenic viral genes, (thus, inhibiting virus replication) and for use as an instrument for functional genomics. RNAi can target specific genes and control their expression. The problem with escape virus mutants has an alternative solution via a method, based on the replication complex of the bacteriophage phi 6. The technology produces a pool of siRNAs targeted to a specific gene region, which overlaps and silences the target (from any eukaryotic organism) whether a mutation or recombination appear.

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Microscopic and Molecular Genetic Methods in the Detection of Bacterial Vaginosis

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Abstract

Every obstetrician or microbiologist encounters bacterial vaginosis (BV) in his/her practice. 50% of the cases are asymptomatic. The percentage of affected women varies. The aim of this study is interpretation of microscopic and molecular diagnostic techniques for diagnosing fastidious microorganisms associated with BV. 234 women were tested. Vaginal secretions were collected, after medical history and detailed gynecological status were completed. Smears were prepared and stained by Gram. Identification of fastidious *Gardnerella vaginalis*, *Atopobium vaginae*, *Megasphaera* sp. type 1, and BV associated bacterium type 2 (*BVAB2*) was performed by PCR. 112 of the 234 tested women had microscopic evidence of some vaginal problem. 65 of them, had anaerobic infection found on the basis of the microscopic data. Materials from women suspected for anaerobic infection were subjected to PCR for proving of specific anaerobes. In 63 patients *G. vaginalis* was found, in 27- *A. vaginae*, in 18 - *Megasphaera* sp. type 1, and in 15 - *BVAB2*. The detected anaerobe in 25 swabs was single. Combination of several pathogens was demonstrated in 40 women. Gram stain is a fast, easily performed, and specific method for diagnosing BV. Methodology limitation is the inability to distinguish visually the different microorganisms involved in the condition. The PCR method provides possibilities for the sensitive, highly specific, and rapid identification of bacteria-specific markers for BV. Accurate determination of microorganisms involved in this syndrome provides possibilities for an adequate and complete treatment of the women affected.

Key words: Bacterial vaginosis, Gram stain, polymerase chain reaction, *Gardnerella vaginalis*, *Atopobium vaginae*, *Megasphaera* sp. type 1 and *BVAB2*;

Резюме

Всеки гинеколог и микробиолог срещат в практиката си бактериална вагиноза. В 50% от случаите състоянието е безсимптомно. Процентът на засегнатите жени варира. Цел на проучването е интерпретация на микроскопските и молекулярно диагностичните методи за доказване на трудно култивирани причинители на БВ. Изследвани са вагиналните секрети на 234 жени, след снемане на анамнеза и статус. Изготвени бяха натривки, оцветени по Грам. Бе проведена идентификация на *Gardnerella vaginalis*, *Atopobium vaginae*, *Megasphaera* type 1 и *BVAB2* чрез PCR. От 234 изследвани жени при 112 се установиха микроскопски данни за вагинален проблем. От тях при 65 въз основа на натривката бяха получени данни за анаеробна инфекция и съответно проведена PCR за доказване на конкретни анаеробни причинители. Получени резултати: при 63 пациентките бе доказано наличието на *G. vaginalis*, при 27 на *Atopobium* spp., при 18 на *Megasphaera* type 1 и при 15 на *BVAB2*. При 25 причинителят бе един. Коинфекция бе доказана при 40 от пациентките. Оцветяването по Грам е бърз, лесен и специфичен метод за диагностика на БВ. Ограничение е невъзможността за визуално разграничаване на микроорганизмите, въввлечени в етиопатогенезата на състоянието. PCR позволява чувствително, високо специфично и бързо определяне на бактериите-маркери, характерни за БВ. Точното определяне на участващите в БВ микроорганизми дава възможност за адекватна терапия на засегнатите жени.

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Introduction

Bacterial vaginosis (BV) is an infectious, non-inflammatory, polymicrobial syndrome with yet undetermined etiology and pathogenesis that affects the lower portions of the genital tract in women (Sobel, 1997). There is currently no test that is reliable to the extent of providing a correct diagnosis. That is why, a lot of scientific teams continue to search for methods and combinations thereof, which can be relied upon to be specific and sensitive, and to minimize possible false diagnoses, which is also the aim of the present study.

Materials and Methods

A total of 234 vaginal secretions of women were examined in the age range from 18 to 64 years. At first, each woman signed an informed consent. After thorough medical history was taken and detailed gynecological status was completed, most common subjective complaints found were vaginal discharge and discomfort in genital area. Two cotton tips were used for obtaining the smear from the lateral side of the vagina, placing the secretion into Aimes transport medium for culturing.

The first swab was used for preparing Gram stain. The second one was used for DNA extraction and molecular diagnostic examination of *G. vaginalis*, *A. vaginae*, *Megasphaera* sp. type 1 and *BVAB2*. The extraction was performed with commercial kit DNA-Sorb-A (Sacace Biotechnologies Srl, Italy) in compliance with the manufacturer's instructions.

Purified DNA of the quantity of 2.5 µl was used for PCR reactions. The total reaction volume was 25 µl. Specific primer pairs providing amplification were used in the reaction with respective product size: 207 base pairs for *G. vaginalis*, 597 base pairs for *A. vaginae*, 211 base pairs for *Megasphaera* sp. type 1, and 406 base pairs for *BVAB2*. The amplification protocol methodology for proving *BVAB2*, *A. vaginae*, and *Megasphaera* sp., was identical; it was as follows: initial denaturation (95°C, 10 min), followed by 40 cycles of denaturation (95°C, 30 sec), annealing (55°C, 30 sec), and extension (72°C, 30 sec), with a final elongation of the chain under conditions of 72°C for 7 min. The protocol for *G. vaginalis* was as follows: initial denaturation (95°C, 10 min), followed by 40 cycles of denaturation (95°C, 30sec), annealing (62°C, 30 sec), and extension (72°C, 30 sec), with a final extension of the chain under conditions of 72°C for 7 min.

Results

No microscopic evidence of disease was found in 122 (52%) of all 234 women studied. The remaining 112 (48%) women had microscopic evidence of some vaginal problem. 65 (28%) of them were suspect, based on the smear anaerobic infection. These materials were subjected to DNA extraction, and subsequent polymerase chain reaction was performed for detection of any anaerobic bacteria that were possible causative agents of BV. The results obtained were as follows: 63 of the patients demonstrated presence of *G. vaginalis* (96.9%), 27 (41.5%) - of *A. vaginae*, 18 (27.7%) - of *Megasphaera* sp. type 1, and 15 (23.1%) - of *BVAB2*. Only one causative agent was detected in 25 (38%) smears. Coinfections were demonstrated in 40 (62%) tested swabs.

Discussion

BV disease was first described in 1955 by Gardner and Dukes as rarely occurring watery, homogeneous discharge with unpleasant odour (Spiegel, 1991). Weström and co-authors introduce the concept BV in 1984. The International Statistical Classification of Diseases and Related Health Problems (10th Revision Version) for 2006 does not emit BV as an independent disease. At this stage, it is statistically identified under the heading of non-inflammatory diseases of the vagina. According to the World Health Organization recommendations from 2005, BV relates to endogenous infections affecting the reproductive tract in humans.

The different reasons that may lead to this condition still remain unexplained. Various studies demonstrated the conditions that could provoke BV: promiscuity or having a new partner; use of intrauterine contraception; frequent vagina washes, etc. It was found that women cannot become infected with BV when using the toilet, swimming pools, or mutual bed linen. Studies have demonstrated that women without active sexual life rarely develop BV.

In healthy women, vaginal ecosystem is balanced due to different bacteria (Zhou *et al.*, 2004; Sobel, 1999): *Staphylococcus*, *Streptococcus*, *Enterococcus*, *Corynebacterium*, *Escherichia*, *Ureaplasma*, *Mycoplasma*, *Peptostreptococcus*, *Gardnerella*, *Bacteroides*, *Veillonella*, *Bifidobacterium*, and *Candida* (Marrazzo *et al.*, 2002). The balance is maintained mainly by representatives of genus *Lactobacillus*, which convert glycogen in the epithelial cells of the vagina into lactic acid, through the production of hydrogen peroxide, and

provide acidic pH in the vagina. The acidic environment of the vagina inhibits the development of pathogenic and commensal microflora. In addition, the normal flora of the vagina synthesizes antimicrobial peptides that ensure the balance and provide prevention from invasion of atypical microorganisms (Hiller, 1998). Despite these and other defensive mechanisms, characteristic of the vaginal mucosa, each woman, at least once in her life, obtains such an infection. Even repeated (recurrent) infections could be registered in some 5-10% of the female population.

In more than 50% of the cases of BV no characteristic signs are detected. The condition is determined as asymptomatic bacterial vaginosis and requires the use of microscopic and molecular biological methods for the diagnosis specification. Many countries have developed screening BV programs especially for young women in order to prevent possible health complications such as pelvic inflammatory disease (PID), premature birth, coinfections with other sexually transmitted pathogens, etc.

The existence of imbalance in the vaginal ecosystem is typical for BV. Another feature is the abruptly reduced number of representatives of genus *Lactobacillus* (especially *L. acidophilus*). As a result, change in the pH occurs and proliferation of anaerobic bacteria such as *Ureaplasma*, *Mycoplasma*, *Gardnerella*, *Mobiluncus* spp., *Atopobium* sp., *Megasphaera* sp. type1, *BVAB1-3*, *Leptotrichia*, etc., is stimulated. The amount of bacteria in volume of 1 ml from 10^6 (in healthy woman) typically reaches amounts of 10^{13} . These organisms produce a number of organic products that lead to the exfoliation to the vaginal mucosa. It is interesting that more than 50% of women with such changes in the vagina have no presence of any clinical manifestation.

The microbiological diagnosis of BV is a challenge. That is due to the following reasons: the large amount and variety of bacterial etiologic agents; the detection of new species recently accepted as involved in the pathogenetic process; the lack of clear bacterial markers for the condition, etc. For example, one of the bacterial pathogens for years associated with the etiology of BV - *G. vaginalis*, was isolated in 83% - 94% of women with clinical symptoms, but is also evidenced in 36% - 55% among the healthy female population or, more precisely, among women without clinical signs (Hiller, 1993). Another example is *A. vaginae*, also isolated from materials of patients with complaints,

but also from women with regular gynecological examination without any symptoms.

Therefore, the correct detection of that medical condition requires a complex approach in the diagnostic algorithm of BV. There are two main approaches - clinical criteria and application of laboratory tests.

The clinical approach is based on the so-called Amsel (Pheifer *et al.*, 1978, Amsel *et al.*, 1983) criteria, that include the following: vaginal pH > 4.5; thin, gray, and homogenous discharge; positive whiff test - in addition to the mucus of 10% KOH, smell of "amines or fish" occurs; demonstration of clue cells on a saline smear. The presence of 3 of the 4 criteria is indicative of BV (Amsel *et al.*, 1983).

The laboratory tests include preparation of native smears or coloring with Gram, methylene blue, or PAP staining, plus one of the next methods- culturing with subsequent identification of the species; molecular genetic methods; liquid-gas chromatography.

Methods, that are still in investigation stage and, however, are already embarked on the practice in some countries, are:

OSOM® BVBlue® chromogen system- a diagnostic test, which enables determining in vaginal secretions of the presence and level of the enzyme sialidase. It was found that this enzyme is produced by a number of bacteria such as *Gardnerella*, *Bacteroides*, *Prevotella*, and *Mobiluncus*, all associated in the pathogenesis of BV. The sensitivity of the method is 88% - 94%; the specificity amounts to 91% - 98% (Myziuk *et al.*, 2003; Sumeksri *et al.*, 2005; Bradshaw *et al.*, 2005).

FemExam G. vaginalis PIP Activity is another type of test. It detects proline aminopeptidase activity of the anaerobic bacteria, especially in *G. vaginalis*. The sensitivity of the test is 89% - 92%, and the specificity is 94% - 96% (Nelson *et al.*, 1994; Calderón *et al.*, 1997).

FemExam pH - allows the determination of pH and the presence of trimethylamine (Gutman *et al.*, 2005; West *et al.*, 2003).

Different quantitative PCRs for *G. vaginalis*, *A. vaginae*, and other bacterial participants in the pathogenesis of BV are at some stage of development (Menard *et al.*, 2008; Cartwright *et al.*, 2012). At least 17 species undergo research.

Fluorescence in situ hybridization (FISH) is applied for the analysis of urine, wherein BV is determined as biofilm desquamation of the epithelium of the vaginal mucosa in sedimentation (Swidsinski *et al.*, 2010).

In the present study, BV was determined using the combination swab stained by Gram's method, and PCR for detection of *G. vaginalis*, *A. vaginae*, *Megasphaera* sp. type 1 and *BVAB2*.

Culture method with subsequent identification was not used due to the fact that the method process is laborious and time-consuming. On the other hand, the presence of fastidious bacteria such as *Atopobium* spp., *Megasphaera* sp. type 1, *BVAB 1-3*, *Leptotrichia*, *Ureaplasma* and *Mycoplasma* cannot be subjected to cultivation and, thus, it limits the application of the method. Their detection is crucial for adequate therapy. It is due to the fact that most of the *Atopobium* spp. strains are resistant to treatment with metronidazole, frequently used in the therapy of BV. When resistance is present recurrent forms of BV occur.

In this study the prepared and stained by Gram's method smears were assessed, based on a standard scoring system (Tab. 1). The diagnostic criteria of this scoring system were introduced by Spiegel et al and later modified by Nugent *et al.* (Spiegel, 1991). The slides were observed with immersion (1000 × magnification) and were evaluated over 20 visual fields for the following types of bacteria:

- long Gram-positive rods (*Lactobacillus* spp.)
- small Gram-variable rods (*Gardnerella vaginalis*)
- small Gram-negative rods (*Bacteroides* spp.)
- curved Gram-variable rods (*Mobiluncus* spp.)

Gram-positive cocci were not a part of the scoring system.

When Nugent Score system is applied it is important to pay attention on the following: the number of polymorphonuclear leukocytes in BV has to be low (Holmes *et al.*, 1983); and the „clue cells” are very specific BV indicator, if they are more than 20% of the epithelial cells on the smear (Eshenbach *et al.*, 1988).

PCR is one of the main methods by which accurate and quick identification of bacterial markers for BV could be performed. It is important to determine correctly and detect all the microorganisms involved in the etiopathogenesis of BV. This will allow appropriate treatment of the condition. The amplification technologies are characterized by high specificity and sensitivity (Ferris *et al.*, 2004). The presence of different microorganisms does not suppress the test performance. Even more coinfections could be detected, based on the reagents included in the reaction system. Advantage of the latter method is the possibility of using different types

of biological samples comprising the already dead microorganisms in them. This eliminates the problem of the great instability of some of the microorganisms associated with BV. Many PCR techniques have been developed, some of which were used in this study.

No microscopic evidence of the disease was found in 122 (52%) of all of the 234 examined women. Microscopic evidence of vaginal problem was detected in the remaining 112 (48%) women. In 65 (28%) of the latter 112 women, anaerobic infection was suspected, based on Gram stained smear. These materials were subjected to DNA extraction and subsequent polymerase chain reaction for detection of anaerobic bacteria. The obtained results were as follows: *G. vaginalis* was found in 63 smears (96.9%), *A. vaginae* in 27 (41.5%), *Megasphaera* sp. type 1 in 18 (27.7%), and *BVAB2* in 15 (23.1%). 25 (38%) materials had only one causative agent. Coinfections by different pathogens was demonstrated in 40 (62%) vaginal swabs.

Conclusions

Gram staining is a quick, cheap, and sensitive method for the detection of BV. It allows obtaining of information for “clue cells”, lactobacilli, and other microflora, presence or absence of polymorphonuclear leukocytes. Disadvantage of the methodology is its inability of visual differentiation between the microorganisms involved in the etiopathogenesis of the condition. The method is subjective, and requires experience and competence of the observer. The PCR provides possibilities for the sensitive, highly specific, and rapid identification of bacteria-markers of BV. Accurate detection of the involved in BV microorganisms provides possibilities for an adequate treatment of the affected women.

Acknowledgements

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Table 1. Nugent Score (a Gram stained scoring system) for evaluation of bacterial vaginosis

Score	Morphotype		
	<i>Lactobacilli</i> Gram-positive rods	<i>Gardnerella</i> Gram-variable rods	<i>Mobiluncus</i> Gram-variable rods
0	> 30	0	0
1	5–30	< 1	1–5
2	1–4	1–4	> 5
3	< 1	5–30	–
4	0	> 30	–

Interpretation of the Nugent Score system: 0 - 3 - normal; 4-6 - intermediate form; 7-10 - bacterial vaginosis; „clue cells“ - with more than 20% of the epithelial cells.

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A Post-Operative Infection Caused by *Bacillus circulans* and *Paenibacillus macerans* in a Patient with Brain Tumour

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Abstract

We report a case of post-operative infection due to rarely isolated bacteria - *Bacillus circulans* and *Paenibacillus macerans* - in a 49-year-old woman following resection of a tuberculum sellae meningioma. The two bacterial pathogens were isolated from a cerebrospinal fluid specimen. These bacteria are opportunistic pathogens, which have often been ruled out as culture contaminants when isolated from clinical material.

Key words: neurosurgical infection, *B. circulans*, *P. macerans*

Резюме

В статията се описва случай на пост-оперативна инфекция, причинена от рядко изолирани бактерии - *Bacillus circulans* и *Paenibacillus macerans* в 49-годишна жена след резекция на менангиома в областта на tuberculum sellae. Двата бактериални причинители бяха изолирани от цереброспинална течност на пациента. Диференцираните бактерии са опортюнистични причинители, които често се приемат за замърсители на посевките или клиничния материал.

Introduction

Health care-associated (or nosocomial) infections represent one of the most common complications of health care delivery. Between 5 and 10% of patients admitted to acute care hospitals acquire an infection during their hospital stay. One of the major site infections among nosocomial infections in hospitals are surgical site infection (SSI). SSI increase hospital expenses and length of stay, which cost the health care system billions of dollars annually (Logan *et al.*, 2011).

Reports of infections with non-*Bacillus cereus* group species are comparatively rare (Noskin *et al.*, 2001). A few reports of bacteremia, brain abscess, peritonitis, and meningitis due to *Bacillus* spp., mostly in immunocompromised patients, have been published in the literature (Berry *et al.*, 2002; Bert *et al.*, 1995; Galanos *et al.*, 2003; Krause *et al.*, 1999). All *Paenibacillus* species bacteria were originally classified as part of the *Bacillus* genus (Teng *et al.*, 2003). This report describes a case of SSI with two rarely isolated *Bacillus* species in a patient following cranial surgery for meningioma.

Case Report

A 49 year-old woman underwent frontotemporal craniotomy in Neurosurgical Clinic for the treatment of tuberculum sellae meningioma. After resection of the meningioma, surgeons used duraplasty by applying fibrin glue as a dural sealant; a postoperative drainage tube was also added. Ten days after the operation patient began to experience frontal headaches, hypothalamic symptom-complex, giddiness, and became increasingly disoriented. Additionally, she had moderate paresis of the left leg, and electrolytic disorders. A cranial computerized tomography scan showed a small ischemia in the area of caudate nucleus (caput) in the right hemisphere, and data for intracranial postoperative infectious alterations in the wound. A lumbar puncture was performed, yielding xanthochromic cerebrospinal fluid (CSF), and its laboratory examination showed: increase of common proteins - 0.84 g/l; decrease of glucose (5.24 mmol/l) in comparison with its level in the serum (10.63 mmol/l). The patient's white blood cell and C-reactive protein (79.41 mg/L) levels became rap-

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idly elevated. A CSF sample was taken for microbiological analysis, and empirical antibiotic treatment was initiated with ceftriaxone plus amikacin. The antibiotic regimen was changed to intravenous amoxicillin-clavulanic acid five days later. The patient recovered well and was discharged four weeks after the brain tumor surgery.

The CSF specimen was centrifuged and Gram staining of its sediment was performed. Additionally, the sediment was cultured on 5% sheep blood agar, chocolate agar, and thioglycollate broth. Direct Gram staining of the CSF revealed the presence of polymorphonuclear leukocytes and round-ended Gram (+) rods. The overnight cultures yielded a growth of two types of colonies which were sporulating Gram (+) rods. They were identified as *Bacillus circulans* and *Paenibacillus macerans* via BBL Crystal™ GP identification panel (Becton Dickinson Company Ltd.) and traditional routine biochemical tests following the methods given in the Manual of Clinical Microbiology (Logan *et al.*, 2011).

Antimicrobial susceptibility was examined by broth microdilution method as recommended by the Clinical and Laboratory Standards Institute (CLSI) guidelines (CLSI, 2010), using commercially prepared panel CMP2IHM Gram (+) (Sensititre, Trek Diagnostic Systems Ltd., UK). The CLSI interpretive standards, established for Staphylococci, were used since no interpretive standards have been established for *Bacillus* spp. (CLSI, 2010).

The microbiological characteristics of the two *Bacillus* spp. isolates were as follows: The colonies of *B. circulans* were spreading on blood agar, sporulating with subterminal spore, distending the cell. It grows on sheep blood agar as non-hemolytic, grey, and sticky to agar (adherent colonies). It is motile, produces catalase, and does not produce any acid from arabinose and cytochrome oxidase; BBL Crystal™ GP identification panel showed that it was 99,9% *B. circulans*. The strain was resistant to penicillin, ceftriaxone, chloramphenicol, and clindamycin, and was sensitive to amoxicillin-clavulanic acid, gentamicin, amikacin, vancomycin, levofloxacin, and meropenem. The isolate *P. macerans* was also sporulating with subterminal spore, not distending the cell. It grows as non-hemolytic, large (3-4 mm), off-white, and butyrous (in consistency) colonies; the species was motile, produces catalase and acid from arabinose and does not produce cytochrome oxidase; BBL Crystal GP panel showed that it was 99,3% *P. macerans*. This isolate was resistant to penicillin, ceftriaxone, and clinda-

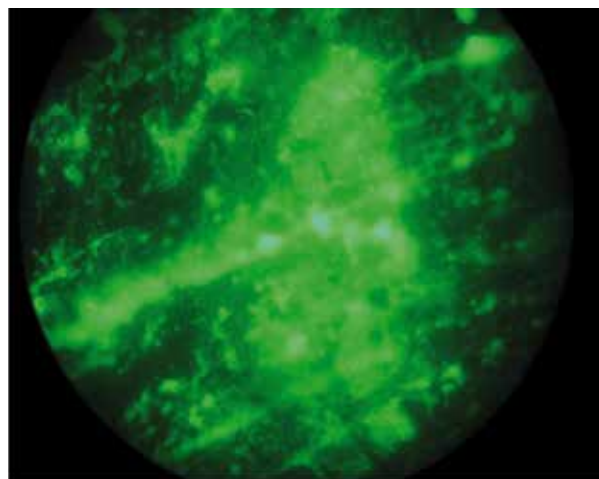


Fig. 1. *Bacillus circulans* - an indirect fluorescent antibody test.

mycin, and was sensitive to amoxicillin-clavulanic acid, gentamicin, amikacin, vancomycin, chloramphenicol, levofloxacin, and meropenem.

Additionally, two serologic tests were performed 11 (the first serologic test) and 23 days (the second serologic test) after the onset of post-operative infection in patient, with cultures on solid media for both bacillary strains that were isolated from CSF. We performed an indirect fluorescent antibody test (FA) to measure the antibody levels of *Bacillus* spp. bacteria, as shown in Fig. 1 and Fig. 2.

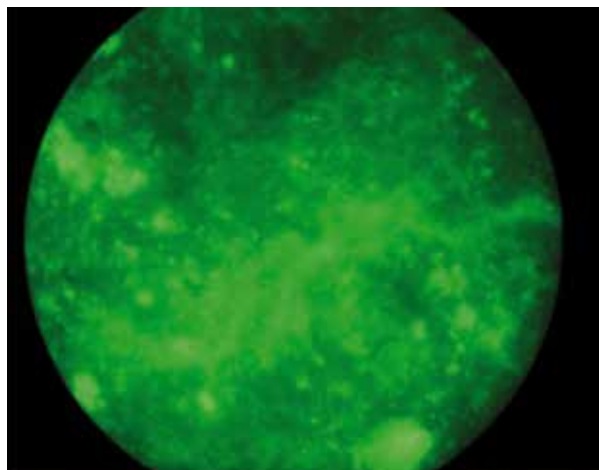


Fig. 2. *Paenibacillus macerans* - an indirect fluorescent antibody test

Increasing antibody titers were detected: 16 from the first serologic test and 64 from the second one for *B. circulans*, and, respectively, 8 from the first test and 32 from the second for *P. macerans*, after twelve days.

Making an attempt at determining the mechanism of β -lactam resistance in the both *Bacillus* species, we performed β -lactamase testing using

two methods: a chromogenic cephalosporin test with nitrocephin paper disks (Cefinase, BBL), and Crude enzyme test with homogenates obtained by ultrasound distraction of the cells. The Crude test was performed with 5 µl of the cells and 30 µl nitrocephin solutions (500 mg/L). Beta-lactamase production has not been detected by these methods.

Discussion

Bacillus spp. are aerobic Gram (+), spore-forming rods, normally found in soil, water, and decomposing organic matter (Logan *et al.*, 2011). Since *Bacillus* spp. are sometimes laboratory contaminants, isolation of the organisms from clinical specimens of sterile sites does not indicate infection unless they are detected in multiple sets (Galanos *et al.*, 2003). Our patient was considered to have a genuine, post-operative infection, despite that bacteria were isolated from CSF sample, and that we had clinical and laboratory data for central nervous system infection 10 days after operation (surgical excision of a brain tumor). These isolates were considered to be responsible for the infectious process, because direct Gram staining of the CSF demonstrated spore-forming Gram (+) bacilli, and increasing serum antibodies in double samples against both isolated bacteria were detected. The two serologic FA tests on patient for detection of antibodies can confirm the clinical diagnosis after cultures were obtained. These *Bacillus* spp. are less pyogenic microorganisms, which was the reason for late onset of this postoperative wound infection. The two bacillary strains are environmental organisms with relatively low pathogenicity, and they were probably introduced intraoperatively (the surgeons supposed it occurred by fibrin glue used for plastic surgery of dura). However, we could not establish the source of infection because we took the material for culturing of this fibrin glue from a new batch.

A few reports of infections due to *Bacillus* spp., mostly in immunocompromised individuals, have been published in the literature. *B. circulans* has been described as a cause of intravenous catheter-related bacteremia, cerebrospinal fluid shunt infections, prosthetic heart valve endocarditis, wound and joint infections (Logan *et al.*, 2011, Galanos *et al.*, 2003). *P. macerans* was due to pseudobacteremia (Noskin *et al.*, 2001, Teng *et al.*, 2003).

Most *Bacillus* spp. are sensitive to penicillin and ceftriaxone, which are a kind of empiri-

cal therapy for central nervous system infections (Weber *et al.*, 1988), but in our case *B. circulans* and *P. macerans* were resistant, and we had improvement in our patient after introduction of the intravenous antibiotics amikacin and later amoxicillin-clavulanic acid. We could not prove β -lactamase enzymes in both *B. circulans* and *P. macerans* species, but our hypotheses are that mechanisms of β -lactam resistance in *Bacillus circulans* and *Paenibacillus* are based on membrane-associated enzymes that have been rarely reported in other *Bacillus* spp. (Logan *et al.*, 2011)

Prevention of post-operative nosocomial infections is unfortunately not achieved even with the antibiotics used for prophylaxis. The most important aspect of prevention of nosocomial infections is strictly observed aseptic working.

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Effect of Cadmium Ions on the Fungal Strain *Aspergillus fumigatus* 3₂ Isolated from Metal Polluted Soil

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Abstract

The fungal strain *Aspergillus fumigatus* 3₂ was isolated from metal polluted soil (around the tailing Vljakov vrah, Bulgaria). Effect of enhanced concentrations of cadmium ions (redox-inactive metal) on the growth and morphology, as well as the participation of oxidative stress in cadmium-induced toxicity, was reported. *A. fumigatus* demonstrated a high tolerance to cadmium, exhibiting remarkable growth in liquid and agar media. High metal concentrations affected fungal morphology and caused oxidative stress events such as changes in the level of reserve carbohydrates and oxidative damaged proteins. A sharp increase in trehalose content and accelerated consumption of glycogen in the presence of cadmium ions at concentration above 5 µg/ml were detected. In addition, a decrease in carbonylated proteins was measured, particularly pronounced at high concentrations (70 and 100 µg/ml). Cadmium ions exposure with 5, 20, and 50 µg/ml resulted in enhanced superoxide dismutase (SOD) level, but the higher concentrations (70 and 100 µg/ml) significantly inhibited this activity. Continuous decrease was also observed for catalase (CAT) activity. Probably, at higher concentrations of cadmium ions antioxidant enzymatic defence does not seem to be a major mechanism of cadmium tolerance in the strain *A. fumigatus*.

Key words: heavy metals, filamentous fungi, oxidative stress, biomarkers, antioxidant enzymes

Резюме

Щам *Aspergillus fumigatus* 3₂ е изолиран от почви в района на медни мини Влайков връх (България), замърсени с тежки метали. Проучен е ефектът на повишаващи се концентрации кадмиеви йони върху растежа и морфологията на моделния организъм, както и участието на оксидативния стрес в метал-индуцираната токсичност. *A. fumigatus* проявява растеж в течна и агарова среда в присъствие на кадмиеви йони до 200 мкг/мл, което доказва неговата висока толерантност. Едновременно с това се наблюдават изменения в морфологията на колонииите. Третирането на културата с повишаващи се концентрации метални йони предизвиква промени в нивата на резервните въглехидрати и оксидативно увредените белтъци, което е указание за проявата на оксидативен стрес. Установено е рязко увеличение в съдържанието на трехалозата и ускорено усвояване на гликогена в присъствието на кадмиеви йони в концентрация над 5 мкг/мл. Наблюдава се понижаване в количеството на белтъците, съдържащи карбонилни групи, особено силно изразено при високите концентрации (70 и 100 мкг/мл). Докато във вариантите с 5, 20 и 50 мкг/мл се наблюдава повишена активност на ензима супероксид дисмутаза (СОД), то при концентрации 70 и 100 мкг/мл тази активност значително се понижава. Отбелязва се и понижаване активността на ензима каталаза във всички третирани култури. Вероятно, антиоксидантната ензимна защита не е част от основния механизъм за толерантност на *A. fumigatus* към високи концентрации на кадмиеви йони.

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Introduction

Heavy metals are present in soils as free metal ions, soluble metal complexes (sequestered to ligands), exchangeable metal ions, organically bound metals, precipitated or insoluble compounds such as oxides, carbonates, and hydroxides, or they may form part of the structure of silicate materials (indigenous soil content) (Leyval *et al.*, 1997). Some metals that have received more attention are mercury, cadmium, and lead, because of their highly toxic properties and their effects on the environment and the living organisms (see Singare *et al.*, 2012).

Cadmium is a very toxic metal - a significant environmental pollutant. This heavy metal or a metal trace element, is dispersed in natural and agricultural environments principally through human activities such as mining, refining, municipal waste incinerators, and fossil fuel combustion sources (Wagner, 1993), as well as natural rock mineralization processes (Sanità di Toppi and Gabrielli, 1999). Cadmium presence into agricultural soils resulted in the application of phosphatic fertilizers (Williams and David, 1976; McLaughlin *et al.*, 2000), soil amendments with municipal sewage sludges, and atmospheric deposition (Wagner, 1993; Weissenhorn and Leyval, 1995).

In naturally polluted environments, the microbe's response to heavy metals toxicity depends on the concentration and the availability of metals, and on the action of factors such as the type of metal, the nature of medium and microbial species (Hassen *et al.*, 1998). One of the major mechanisms behind heavy metal toxicity includes production of reactive oxygen species (ROS). These highly reactive ROS can interact with various cellular components leading to oxidative damage of all cellular macromolecules. To minimize the damaging effects of ROS, aerobic organisms evolved both non-enzymatic and enzymatic antioxidant defense systems. Microorganisms have been shown to possess an ability to survive by adapting or mutating at high concentrations of toxic heavy metals. Fungi are also known to tolerate heavy metals (Gavrilescu, 2004; Baldrian, 2003).

The response of microorganisms towards toxic heavy metals is significant due to the interest they represent in the reclamation of polluted sites. Living organisms exposed environmentally to high metal concentrations follow various mechanisms to counter potential toxicity. Recent research indicates that cadmium induces oxidative damage in cells, and alterations in the activities of antioxidant enzymes (Yildirim and Asma, 2010). Although it was

described that heavy metals are extremely toxic to a lot of living cells, little is known about the defence responses to metal-induced oxidative stress at subcellular level. We are interested in the way the fungal cell counteracts cadmium toxicity. The aim of this recent study is to investigate the cell response of the filamentous fungus, isolated from metal polluted soils, against cadmium. Changes in the fungal morphology and physiology as a result of cadmium ions treatment were observed. The role of antioxidant enzyme system in the cell response against cadmium toxicity was investigated.

Materials and Methods

Microorganism and Culture Conditions

The investigation was performed with filamentous fungus *Aspergillus fumigatus* isolated from a soil sample taken from a metal polluted region with strong industrial activity in the tailing Vlahkov vrah near Pazardzhik (Bulgaria). Submerged cultivation was performed in 500 ml Erlenmeyer flasks for 72 hours. Composition of the seed and production media, and the culture conditions were as previously described (Krumova *et al.*, 2009). For investigation of the effect of different cadmium concentrations, cultures were incubated with different concentrations of cadmium chloride, in order to achieve 5, 20, 50, 70, and 100 µg/ml of cadmium ions, added at the beginning of cultivation. Results were evaluated from repeated experiments using three parallel runs.

Macroscopic Study

To monitor the metal-resistance of the fungal strain, conidiospores were cultivated in Petri dishes (d = 10 mm) with beer agar, supplemented with various concentrations of cadmium chloride in order to achieve 50, 100, and 200 µg/ml for 7 days at 28°C. The following morphological characteristics were evaluated: colony growth (length and width), presence or absence of aerial mycelium, colony color, presence of wrinkles and furrows, pigment production, etc.

Cell-Free Extract Preparation

The cell-free extract was prepared as described earlier (Angelova *et al.*, 1995). All steps were performed at 0-4°C.

Enzyme Activity Determination

SOD activity was measured in cell-free extracts (CFE) by nitro blue tetrazolium (NBT) reduction (Beauchamp and Fridovich, 1971). One unit of SOD activity was defined as the amount of SOD required for inhibition of the reduction of

NBT by 50% (A_{560}) and was expressed as units per mg protein (U/mg protein). Catalase was assayed by the method of Beers and Sizer (1952) in which the decomposition of hydrogen peroxide was analysed spectrophotometrically at wavelength of 240 nm. One unit of catalase activity was defined as the amount of enzyme that decomposes 1 mmol hydrogen peroxide per minute at an initial hydrogen peroxide concentration of 30 mmol/L, at pH = 7.0 and 25°C. The specific activity is expressed in U/mg protein.

Measurement of Protein Carbonyl Content

Protein oxidative damage was measured spectrophotometrically as protein carbonyl content, using the dinitrophenyl hydrazine (DNPH) binding assay (Levine *et al.*, 1990), slightly modified by Adachi and Ishii (2000).

Determination of Reserve Carbohydrates

In order to determine glycogen and trehalose content, a procedure, previously described by Becker (1978) and Vandecamen *et al.* (1989), and then modified by Parrou *et al.* (1997), was used. Soluble reducing sugars were determined by the Somogyi-Nelson method (Somogyi, 1952).

Other Analytical Methods

Protein was estimated by the Lowry procedure (Lowry, 1952), using crystalline bovine albumin as a standard. Dry weight determination was performed on samples of mycelia harvested throughout the culture period. The culture fluid was filtered through a Whatman filter (Clifton, USA No. 4). The separated mycelia were washed twice with distilled water and dried to a constant weight at 105°C.

Results

Effects of Cadmium on the Colony Growth and Morphology

The effect of cadmium on the growth of *A. fumigatus* 3₂ consisted of three components: the

reduction of radial growth rate, inhibition of formation of conidia, and changes in colony morphology in the presence of heavy metal of up to 200 µg/ml (Table 1). Concentrations of up to 20 µg/ml cadmium ions did not significantly affect the colony growth (data were not shown). However, the exposure to the enhanced concentrations of cadmium ions resulted in a significant reduction in colony diameter in a concentration-dependent manner. At the highest concentrations applied (200 µg/ml), the model strain formed microcolonies.

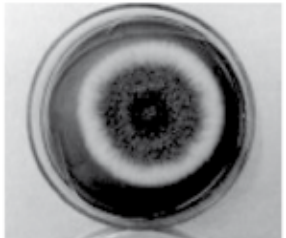
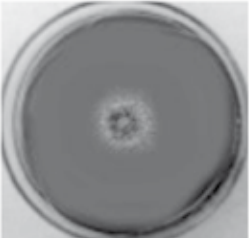
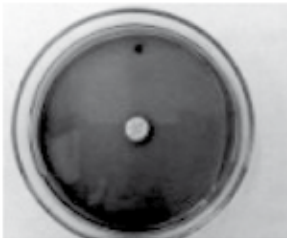
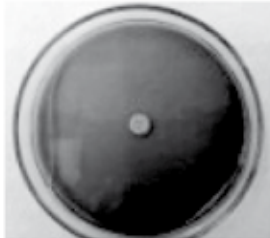
Table 1 also demonstrates the variability of morphological features in the presence of cadmium ions. Several authors have reported the formation of colorful mycelia in the presence of heavy metals on agar media (Darlington and Rauser, 1988). In the present study, changes in colony color from blue-green in the control variant, through yellow (at 50 and 100 µg/ml Cd²⁺), to white (at 200 µg/ml) were observed.

Moreover, surface changes also occur in the presence of cadmium ions. The control variant demonstrated smooth velvety surface, gray-green aerial mycelium, and green substrate mycelium with white, jagged edge without fluid droplets. Presence of cadmium ions leads to forming of yellow velvet colony with streaked surface. With increasing metal concentrations, the culture showed delayed sporulation along with increase in content of cadmium ions.

Effect of Metal Concentration on Biomass Production under Submerged Cultivation

Growth of *A. fumigatus* was studied in relation to enhanced cadmium concentration (5, 20, 50, 70, and 100 µg/ml) under submerged conditions (Fig. 1). The results illustrated that the model strain can grow in the presence of a wide range of cadmium ion concentrations (5 - 100 µg/ml). However, the presence of cadmium ions influences the biomass production by decelerating the rate of growth

Table 1. Morphological changes in model strain in the presence of different concentration Cd ions

Cd ²⁺ concentration [µg/ml]			
Control	50	100	200
			

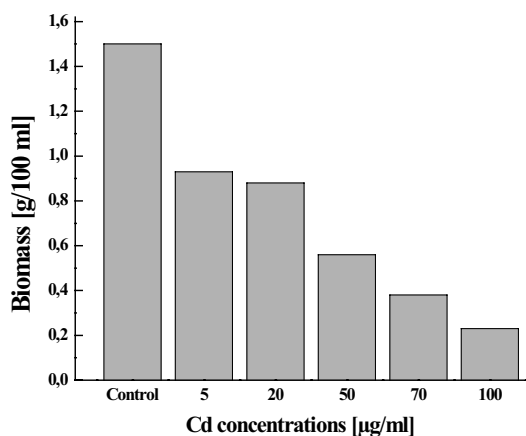


Fig. 1. Biomass content of *A. fumigatus* in presence of different Cd ions concentrations

in comparison with the control medium. A clear trend of cadmium-induced decrease in biomass content in dose-dependent manner was observed. Concentrations of 5 µg/ml led to reduction in biomass by 38%, as compared with the control. Although the enhanced presence of cadmium ions in culture medium caused a significant decrease in dry mass content (about 80% of the control), the strain demonstrated mycelial growth even at 100 µg/ml.

Cadmium-Induced Toxicity and Oxidative Stress Biomarkers

Excess of heavy metals cause toxic effects in several ways, one of them being the excessive production of ROS, which disturb the cellular redox environment causing oxidative stress. To investigate whether cadmium ions interaction with *A. fumigatus* cells causes oxidative stress events, we evaluated the changes of reserve carbohydrate accumulation and oxidative damaged protein content after exposure to enhanced metal concentrations.

Figure 2 demonstrates the effect of cadmium ions on glycogen and trehalose level in the fungal cells. As the main source of carbon- and energy-storage in fungi, glycogen is an important factor for viability under stress conditions.

At low metal concentration (5 µg/ml), a abrupt reduction of the glycogen content was found (by 58% as compared with the control). The same glycogen level was determined in the cultures treated with higher concentrations (20 - 100 µg/ml). Thus, at each concentration, the glycogen accumulation in the fungal cells depends on presence of cadmium ions, but not on their content.

An opposite situation was observed for trehalose response to the metal-induced toxicity (Fig. 2). The interaction of fungal cell with 5 µg/ml cadmium ions did not cause significant change

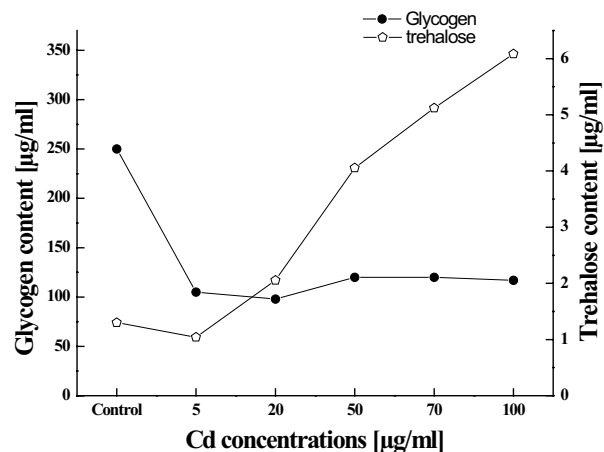


Fig. 2. Effect of enhanced concentrations of Cd ions on glycogen and trehalose accumulation

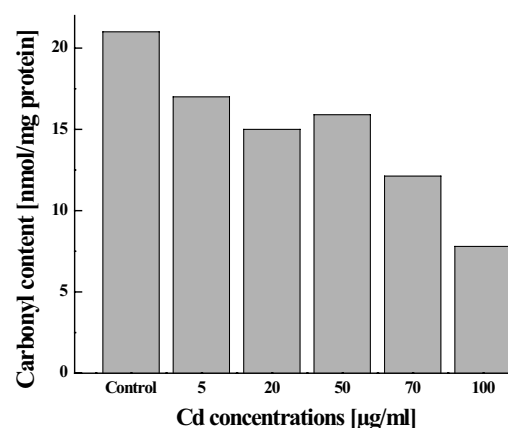


Fig. 3. Level of damaged proteins in the presence of different concentrations of Cd

in trehalose content, but the exposure to higher concentrations resulted in a sharp continuous increase. As seen in the figure, 2.5-fold increase in the trehalose level was found at application of 100 µg/ml cadmium ions.

The usage of protein carbonyl groups as biomarkers of oxidative stress has some advantages in comparison with the measurement of other oxidation products because of the relatively early formation and the relative stability of carbonylated proteins. The carbonyl content in the total proteins extracted from *A. fumigatus* cultures treated by enhanced concentrations of cadmium ions is presented in Fig. 3.

Unexpectedly, a tendency for gradual reduction in protein carbonylation level can be seen. The presence of 5, 20, and 50 µg/ml cadmium ions resulted in about 20 - 28% decrease in oxidative damaged protein content compared to the control. The results showed that at higher concentrations (50 and 70 µg/ml), the protein carbonyl content continued to decrease by 42 - 63% compared to untreated cultures.

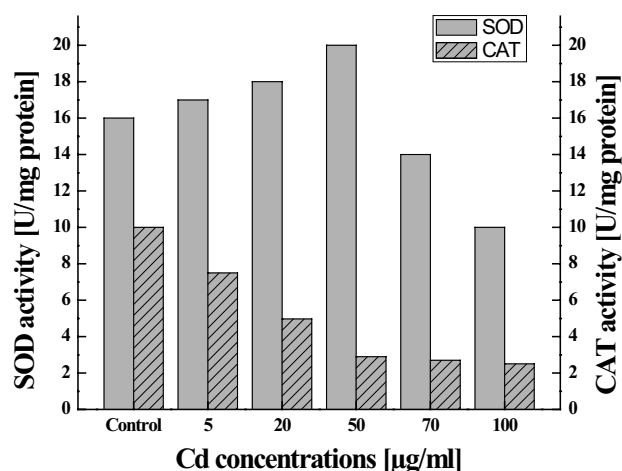


Fig. 4. Enzyme antioxidant activity in presence of Cd ions

Antioxidant Enzyme Activities in Presence of Cadmium Ions

The changes in activity of the main antioxidant enzymes, superoxide dismutase (SOD) and catalase (CAT), in *A. fumigatus* cells, induced by cadmium exposure (from 5 to 100 µg/ml) are presented in Fig. 4.

As compared with the untreated cultures, SOD activity - responsible for the elimination of superoxide radicals in cells - gradually increased after exposure to the concentration of 5, 20, and 50 µg/ml. At cadmium doses of 70 and 100 µg/ml the SOD activity in the fungal cells decreased from 16.22 to 14.16 and 9.97 U/mg protein, respectively. In contrast, CAT activity in treated cultures was significantly lower than that in the control cells. In the presence of 50, 70, and 100 µg/ml cadmium ions only 25% of control activity was measured.

Discussion

Present data confirm that the fungus *A. fumigatus* 3₂ showed a higher tolerance to cadmium ions in comparison with other fungi, reported in the literature (Guelfi *et al.*, 2003; Todorova *et al.*, 2008). This strain exhibited growth at 100 and 200 µg/ml in liquid and agar media, respectively. Similar remarkable potential of growth in agar medium containing cadmium has been published for *Aspergillus versicolor*, *Pisolithus tinctorius*, *Trichoderma* sp., etc. (Mohammadian Fazli *et al.*, 2015; Carrillo-Gonzalez *et al.*, 2012; Joshi *et al.*, 2011). On the other hand, inhibition of growth was also found in both types of cultivation, under conditions of cadmium stress. Growth reduction is a typical response of fungi to the toxicity of heavy metals (Baldrian,

2010). Ramsay *et al.* (1999) have found that the colony extension rates of several fungal species decreased under conditions of cadmium toxicity. Gadd *et al.* (2001) also reported a decrease in radial expansion of *Trichoderma viridae* and *Rhizopus arrhizus* in the presence of heavy metals, including cadmium. The authors noted the importance of glucose concentrations for metal-induced toxicity. *A. fumigatus* 3₂ demonstrated biomass formation under submerged cultivation in the presence of 100 µg/ml cadmium ions that were 20% of the control dry weight. The same concentrations (100 parts per million = 100 µg/ml) inhibited the dry weight of *Fusarium oxysporum* by 85% in comparison with the control (Golubović-Ćurguz *et al.*, 2010). Hassn *et al.* (2014) and Todorova *et al.* (2008) reported a similar trend in growth in the presence of cadmium ions of *Isaria javanica* and *Aspergillus niger* B77, respectively.

Besides the growth, cadmium, in concentration between 50 and 200 µg/ml, causes pronounced morphological aberrations in the *A. fumigatus* cells. This may be direct or indirect result of cadmium effects on cell division, protein synthesis, and cellular organelles such as mitochondria (Trevors *et al.*, 1986). According to Zafar *et al.* (2007), the morphological changes may be due to the vast detoxification/tolerance mechanisms that the fungus uses. Significant differences in pigmentation in *A. fumigatus* colonies were also observed in metal-treated colonies in comparison with the control mycelia. Similar decoloration occurred by increasing the cadmium concentration in medial growth for *A. versicolor*, *Cladosporium* sp., and *Paecilomyces* sp. (Mohammadian Fazli *et al.*, 2015). As it has been previously suggested, the production of pigments in fungal cell is accompanied by precipitation of metal ions on the cell wall (Gruhn and Miller, 1991). Opposite results were reported by Baldrian and Gabriel (2002) about the effect of cadmium on the morphology of *Piptoporus betulinus*. They established that 50, 100, and 250 mM cadmium ions did not cause color changes.

Many studies showed significant increase in ROS content during cadmium exposure that leads to oxidative stress. Cadmium itself is unable to generate free radicals directly, however, indirect formation of ROS involving the superoxide radical and hydroxyl radical has been reported (Aflaine *et al.*, 2015). In addition, the generation of non-radical hydrogen peroxide, which itself, in turn, maybe a significant source of radicals *via* Fenton chemistry, has to be noted. In this mechanism,

cadmium can replace iron and copper in various cytoplasmic and membrane proteins, thus increasing the amount of unbound free or chelated copper and iron ions participating in oxidative stress and cadmium-induced toxicity. Our results confirm the enhanced ROS generation, demonstrating changes of the oxidative stress biomarkers, such as reserve carbohydrates and oxidative damaged proteins. Glycogen and trehalose are the two major reserve carbohydrates in the fungi and can represent up to 25% of the dry cell mass, depending on the environmental conditions (Scebba *et al.*, 2006). The changes in reserve carbohydrate content in fungal cells have been demonstrated for different adverse conditions (Parrou and Francois, 1997b; Ocón *et al.*, 2007; Kostadinova *et al.*, 2012). In the present study, there was a sharp increase in trehalose content of cells, grown in presence of cadmium ions in concentration above 5 µg/ml. This increase is of similar magnitude to the one observed in *Corollospora lacera* and *Monodictys pelagica* after exposure to cadmium ions (Taboski *et al.*, 2005) and *Candida albicans* under conditions of heat shock (Scebba *et al.*, 2006). The accumulation of trehalose has been correlated with higher tolerance to a variety of abiotic stresses (Elbein *et al.*, 2003). Previous research studies have reported that trehalose is necessary for the growth and stress adaptation. It is directly involved in the synthesis of other compounds, energy production, and membrane stabilization, acting as regulators of gene expression and sugar-sensing signal system (see Xie *et al.*, 2014). By rapidly reacting with ROS, trehalose would prevent their reaction with proteins and other cellular constituents (*e.g.* DNA, RNA, or lipids) (Benaroudj *et al.*, 2001; Luo *et al.*, 2008). Furthermore, Benaroudj *et al.* (2001) demonstrated the ability of trehalose to reduce oxidant-induced modifications of proteins and its capacity to prevent protein aggregation.

Glycogen is also known as useful biomarker since its changes are not transient or sensitive to non-toxicant stress. In contrast to the reported increase in glycogen level under stress conditions, our results revealed that cadmium exposure significantly reduced its content in the *A. fumigatus* cells. A similar decrease has also been highlighted in *A. niger* and *Trichosporon cutaneum* exposed to cadmium (Todorova *et al.*, 2008; Lazarova *et al.*, 2014). The decrease in energy content in cells, exposed to cadmium, could be explained by the energetic cost of tolerance, offsetting the stress produced by the toxicant (Sornom *et al.*, 2012). During stress condi-

tions, the cell must regulate the flux of glucose into trehalose generation, glycogen synthesis, pentose phosphate shunt, and glycolysis. The cell may buffer its intracellular glucose levels by the continued flux into and out of its glycogen stores. A progressive glycogenolysis (breakdown of glycogen(n) to glucose-1-phosphate and glycogen(n-1)) in the cells has been found (Emad *et al.*, 2005).

Generally, the level of oxidatively damaged (carbonylated) proteins increases during oxidative stress. This situation indicates that the quantity of generated ROS exceeded the capacity of the antioxidant defensive system. Published data demonstrated that the exposure of different aerobic cells to cadmium ions caused a remarkable increase in carbonyl formation, indicating that cadmium promoted a high protein oxidation (Emad *et al.*, 2005; Aflanie *et al.*, 2015). In contrast, present study illustrated a decrease of carbonylated proteins content in *A. fumigatus* cells treated with cadmium. Similar data have been found for *Trichosporon cutaneum* R57 treated with 5 and 10 mM cadmium sulfate (Lazarova *et al.*, 2014). One possible explanation could be based on both the enhanced degradation of proteins by proteases and aggregation of heavily oxidized proteins. Oxidized proteins serve as better substrates for proteolytic digestion. It has been suggested that protein oxidation could predispose it to ubiquitination, which, in turn, would be a target for proteasomal degradation (Cabisco *et al.*, 2000). At the same time, degradation of oxidized proteins removes potential toxic fragments and provides aminoacids for new protein synthesis (Pena *et al.*, 2008).

The fungal response to cadmium-induced cells includes activation of antioxidant enzymes. SOD and CAT are important components of defensive mechanisms in fungi. SOD catalyzes the dismutation of the superoxide radicals into either ordinary molecular oxygen or hydrogen peroxide. CAT is the most important enzyme for the regulation of intracellular hydrogen peroxide levels (Blokina *et al.*, 2003). Some researchers have indicated the activity of antioxidant enzymes is elevated in fungal cells under cadmium stress (Ott *et al.*, 2002; Todorova *et al.*, 2008; Lazarova *et al.*, 2014). A cadmium-stimulated increase in SOD and CAT activity was also observed in *Aspergillus nidulans* (Guelfi *et al.*, 2003). But, our present results showed that the concentrations of cadmium ions above 50 µg/ml resulted in about 20 or 38% decrease in SOD activity. A significant decrease was also observed for CAT activity. According to Ott *et al.* (2003), the addition of cadmium

resulted in antioxidant enzyme fluctuations relative to controls in fungal cultures. In *Neurospora crassa* cadmium did not induce any changes in SOD level, whilst in *Rhizopogon roseolus* and *S. cerevisiae* cadmium caused reduction in SOD activity (see Guelfi *et al.*, 2003). Similar results have been reported on cadmium-stress response in different plants (Scebba *et al.*, 2006; Zhang *et al.*, 2015). Probably, besides the detoxification function, antioxidant enzyme molecules may also be sensitive targets of cadmium toxicity (Gallego *et al.*, 1996). The reduction in SOD activity can be attributed to the inhibition of enzyme activity by excess hydrogen peroxide content that is a product of accelerated superoxide dismutation (Malar *et al.*, 2014). At the same time, CAT inhibition could be explained by the increased accumulation of hydrogen peroxide. Moreover, induction of stress proteins and/or non-enzymatic antioxidant formation can be included in the defence system (Radhakrishnan, 2010).

Conclusion

Altogether, our results show that *A. fumigatus* 3₂, isolated from polluted soil, contain different mechanisms to cope with toxicity of cadmium ions. High metal concentrations affected the fungal growth and morphology. In addition, exposure to cadmium ions clearly resulted in oxidative stress events such as changes in the level of reserve carbohydrates and oxidative damaged proteins. Our study showed a dose-dependent alteration in the *A. fumigatus* antioxidant status. While at low cadmium concentrations, SOD and CAT provide defence against metal toxicity, the higher cadmium level caused significant inhibition of the activities of both enzymes. Apparently, scavenging of the produced ROS by antioxidative enzymes does not seem to be a major mechanism of cadmium tolerance in the model strain under extreme metal concentrations.

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Investigation of Antioxidant and Antiviral Properties of Geraniol

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Abstract

Geraniol is an acyclic monoterpene alcohol with characteristic rose-like odour. It is an important constituent of Bulgarian *Rosa alba* L. and *Rosa damascena* Mill. essential oils. The purpose of the present study was to investigate antioxidant ability as well to reveal the potential for antiviral activity of geraniol against the replication of viruses belonging to different taxonomic groups and representing important human pathogens. Geraniol significantly depressed the effect of oxidation - it showed good ability to capture 2,2-diphenyl-1-picrylhydrazyl (DPPH) radicals and to inhibit lipid peroxidation in a egg liposomal suspension. Geraniol showed low cytotoxicity toward HEp-2 cells. It was tested *in vitro* for its activity against viruses representing important human pathogens assigned to different taxonomic groups: coxsackievirus B1 (CV-B1) from the *Picornaviridae* family, respiratory syncytial virus (RSV) from the *Paramyxoviridae* family, and influenza virus A/Aichi/68/H3N2 from the *Orthomyxoviridae* family. *In vitro* antiviral effect was examined by the virus cytopathic effect inhibition assay. Geraniol showed antiviral activity only against CVB1 - the ratio of selective index is 3.9. The investigated biological properties of geraniol, including good antioxidant and antiviral activities against some virus families, together with negligible toxicity, warrant further studies to explore the feasibility of formulating geraniol-containing consumer products with health promoting properties.

Key words: geraniol, antioxidant activity, antiviral properties

Резюме

Гераниол е ацикличен монотерпенов алкохол с характерен мирис на роза. Той е важна съставна част от етеричните масла на българската *Rosa alba* L. и *Rosa damascena* Mill. Целта на настоящото проучване е да се изследва антиоксидантната способност, както и да се разкрие потенциала за антивирусна активност на гераниол срещу репликацията на вируси, принадлежащи към различни таксономични групи, които са важни човешки патогени. Гераниол показва добра способност да улавя 2,2-дифенил-1-пикрилхидразил (DPPH) радикали и да инхибира липидната пероксидация в моделна система от яйчени липозоми. Гераниол демонстрира ниска цитотоксичност към HEp-2 клетки. *In vitro* беше тествана неговата активност срещу вируси, които са важни човешки патогени, принадлежащи към различни таксономични групи: Коксаки B1 вирус (CV-B1) от семейство *Picornaviridae*, респираторен синцитиален вирус (RSV) от семейство *Paramyxoviridae* и грипен вирус A/Aichi/68/H3N2 от семейство *Orthomyxoviridae*. Антивирусният ефект беше изследван *in vitro* в постановка на многоциклов ЦПЕ (цитопатичен ефект)-инхибиращ тест. Гераниол показва антивирусно действие само срещу CVB1 - селективният индекс е 3.9. Изследваните биологични свойства на гераниол, сред които са добрата антиоксидантна и антивирусна активност срещу някои вирусни семейства, заедно с незначителната токсичност, налагат провеждането на допълнителни изследвания, за да се проучи приложимостта на гераниол-съдържащите продукти с добри здравословни показатели.

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Introduction

Compounds from natural plants are important sources of drugs against a wide variety of diseases. Geraniol (3,7-dimethylocta-trans-2,6-dien-1-ol) is an acyclic monoterpene alcohol with the chemical formula $C_{10}H_{18}O$. The product referred to as “geraniol” is a mixture of the two cis-trans isomers (Fig. 1) properly named geraniol (*trans*) and nerol (*cis*). Geraniol has characteristic rose-like odour and the taste (at 10 parts per million) is described as sweet floral rose-like, citrus, with fruity, waxy nuances (Burdock, 2010). It is an important constituent of essential oil of ginger, lemon, lime, lavender, nutmeg, orange, rose, etc., an acyclic monoterpene, and the main component of oil of rose, e.g. Bulgarian *Rosa alba* L. and *Rosa damascena* Mill. (Mileva *et al.*, 2014). Geraniol is a fragrance ingredient used in decorative cosmetics, fine fragrances, shampoos, toilet soaps, and other toiletries as well as in non-cosmetic products such as household cleaners and detergents. Its use worldwide is approximately greater than 1 000 metric tones per annum (Lapczynski *et al.*, 2008). In addition, geraniol exhibits various biochemical and pharmacological properties. Researchers have shown geraniol to be an effective plant-based insect repellent (Barnard and Xue, 2004) and its potential as an antimicrobial agent has been highlighted in several studies (Bard *et al.*, 1988). Geraniol exerts *in vitro* and *in vivo* antitumor activity against murine leukemia, hepatoma and melanoma cells (Burke *et al.*, 1997; Yu *et al.*, 1995 a, b). Geraniol is reported to prevent cancer (Carnesecchi *et al.*, 2004).

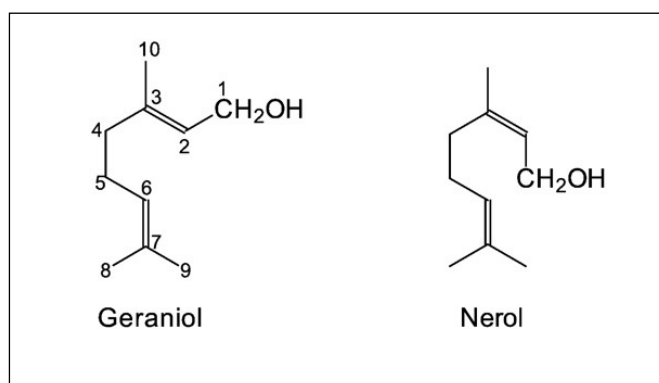


Fig. 1. Chemical structure of geraniol and nerol

The purpose of the present study was to investigate antioxidant activity as well to reveal the potential for antiviral activity of geraniol, against the replication of viruses, belonging to different taxonomic groups and representing important human pathogens.

Materials and Methods

Chemicals Used

All chemicals, standards, solvents, and culture media of high purity (>99%) were purchased from Sigma-Aldrich Chemie GmbH, Merck (Germany), and Givaudan (Switzerland).

DPPH Test

Hydrogen atoms and electron-donating potential of geraniol was measured from the bleaching of the purple-colored ethanol solution of DPPH. The compound was dissolved in ethanol to a concentration of 100 mg.mL⁻¹ stock solutions for the following dilutions. DPPH assay was measured, as follows: freshly prepared ethanolic solution of DPPH (100 mM) was incubated with tested substance in the concentration of 1 to 0.1×10^{-5} mg.mL⁻¹; after incubation for 30 min in the dark, at room temperature, the optical density (OD) was monitored spectrophotometrically at wavelength (λ) of 517 nm. Inhibition of DPPH in percentage (I, %) was calculated as given below:

$$I (\%) = \frac{[(OD \text{ control} - OD \text{ sample}) / (OD \text{ control})] \times 100}$$

IC₅₀ was defined as the quantity of substance necessary to decrease the initial DPPH by 50%. All activities were compared against 2,6-di-tert-butyl-4-methylphenol (BHT) and ascorbic acid, as well popular antioxidants. Data were obtained from the plotted graph scavenging activity of each sample. Lower IC₅₀ value means higher antiradical activity. Each experiment was performed in triplicate and data were presented as a mean of the three values (Singh *et al.*, 2008).

Extraction of Liposomal Suspension

We used a liposomal suspension obtained from phospholipids of egg yolk as lipid rich media, extracted according to Folch *et al.* (1957). After evaporation under vacuum, the chloroform fraction was dissolved in 50 mM potassium-sodium phosphate buffer pH 7.4 (Sigma Chemicals Company Ltd) to a final concentration of 2 mg lipid.mL⁻¹, and vortexed for 10 min. Ultrasonic sonication was performed in Branson ultrasonic bath for 30 min.

Antioxidant Activities in Liposomal Suspension

Antioxidant activities in liposomal suspension were measured by formation of endogenous lipid peroxidation products, reacting with 2-thiobarbituric acid (TBARS), and detected spectrophotometrically ($\lambda = 532$ nm) by the method of Bishayee and Balasubramanian (1971), adapted by Mileva *et*

al. (2000). Briefly, each sample in the test tube contains 1.8 ml liposomal suspension with concentration of 2 mg lipid.mL⁻¹, and 100 µL methanol solutions of compounds to achieve concentrations of 0.01, 0.1, and 1 mg.mL⁻¹, prepared immediately before use. The samples were vigorously stirred and, after pre-incubation for 10 min at 37°C, the induction of lipid peroxidation was initiated by adding of 50 µl Fe²⁺ and 50 µl ascorbic acid to a final concentration of 1 mmol.L⁻¹. After incubation for 30 min at 37°C, the reaction was stopped with 0.5 ml of 15 % trichloroacetic acid and 0.5 ml of 0.67 % thiobarbituric acid. The samples were heated at 100°C for 20 min and cooled in ice. 5 ml of n-butanol was added to each tube - it was vigorously stirred and centrifuged at 1200 × g for 10 min. The amount of TBARS generated in the system was determined from the upper organic layer. The ratio of the absorption at 560 nm for the sample, containing tested substances in different concentration and the same absorption for the controls (without tested substances) in percentage was called antioxidant activity (AOA). The experiments were performed in triplicate.

$$\text{AOA (\%)} = \text{Es/Ec} \times 100\%$$

where Es were content of TBARS, formed in samples, containing tested substances, and Ec were TBARS of the controls (without tested substances). All experiments were performed in triplicate and data were presented as a mean of the three values. As positive control served BHT.

Cells and Viruses

Coxsackievirus B1 (CV-B1) (strain Connecticut) from the *Enterovirus* genus of the *Picornaviridae* virus family, human respiratory syncytial virus A2 (HRSV-A2) from the *Paramyxoviridae* family, were grown in the Hep-2 cell line. Cells and viruses were from the cell culture collection of the *Stephan Angeloff* Institute of the Bulgarian Academy of Sciences, Sofia, Bulgaria. Cell lines were grown in a humidified atmosphere at 37°C and 5% carbon dioxide in Dulbecco's Modified Eagle's Medium (DMEM) (Gibco BRL, Grand Island, NY, USA), in a growth medium containing 5% fetal bovine serum and supplemented with antibiotics (100 IU/mL penicillin, 100 µg/mL streptomycin, and 50 µg/mL gentamycin). When harvesting viruses and performing antiviral assays, maintenance medium was used, in which serum was reduced to 0.5%. Viruses themselves were grown in a humidified atmosphere at 37°C and 5% carbon dioxide.

Cellular Toxicity

Monolayer cell cultures in 96-well plates (Cellstar®, Greiner Bio-one, GmbH, Frickenhausen, Germany) were inoculated with 0.1 mL/well maintenance medium containing different concentrations of the samples in 0.5 lg intervals. On the 48th hour after incubation, they were subjected to the neutral red uptake procedure (Borenfreund E, and J.A. Puerner, 1985), and the 50% cytotoxic concentration (CC₅₀) was calculated. Briefly, after removal of the maintenance medium, which contained the test compound, cells were washed and 0.1 mL fresh maintenance medium, supplemented with 0.005% neutral red dye (Fluka Chemie AG, Buchs, Switzerland), was added to each well and cells were incubated at 37°C for 3 hours. Afterwards, cells were washed once with PBS and 0.15 mL/well desorb solution (1% glacial acetic acid, 49% ethanol, 50% distilled water) was added. After 10 min of mild shaking, the optical density (OD) of each well was read at 540 nm in a microplate reader (Organon Teknika reader 530, Oss, Netherlands). The CC₅₀ value was defined as the concentration of each sample that reduced the absorbance of the treated cells by 50% when compared to the untreated control. The CC₅₀ values were determined by regression analysis.

Antiviral Activity

The virus cytopathic effect (CPE) inhibition assay was used for evaluating the antiviral effects of the samples. Monolayer cells in 96-well plates were inoculated with 0.1 mL virus suspension containing 100 CCID₅₀ (CCID₅₀ is the 50% Cell Culture Infectious Dose which was previously determined by the standard virus titration assay in the respective cell culture). After one hour for virus adsorption (two hours in the case of HRSV-A2), excessive virus was discarded, and cells were inoculated with 0.1 mL of maintenance medium containing different non-toxic concentrations (in 0.5 lg intervals) of the test samples. Then cells were further incubated in a humidified atmosphere at 37°C and 5% carbon dioxide. The CPE was scored daily till the appearance of its maximum in the virus control wells (with no compound in the maintenance medium), that happened usually in 48 hours. Then viable cells were stained according to the neutral red uptake procedure and the percentage of CPE inhibition for each concentration of the test sample was calculated using the following formula:

$$\% \text{ CPE} = [\text{OD test sample} - \text{OD virus control}] / [\text{OD toxicity control} - \text{OD virus control}] \times 100.$$

Table 1. DPPH scavenging activities and AOA in egg liposomal suspension of geraniol and reference standards ascorbic acid and butylated hydroxyl toluene (BHT).

N	Compounds	DPPH IC ₅₀ [μg.L ⁻¹]	AOA (%) 1 mg.mL ⁻¹	AOA (%) 10 mg.mL ⁻¹	AOA (%) 100 mg.mL ⁻¹
1	Geraniol	9.45 ± 0.34	31.13 ± 1.34	24.51 ± 0.34	22.33 ± 0.34
2	BHT	4.03 ± 0.24	34 ± 2.11	27.62 ± 3.11	21.30 ± 1.87
3	Ascorbic acid	3.12 ± 0.37	NT*	NT*	NT*

*NT – non tested; Results are expressed as average ± SD (n=3).

The concentrations that inhibited 50% of the virus-induced CPE, and the 50% inhibitory concentrations (IC₅₀), were determined by regression analysis. The selectivity index (SI) was calculated as the ratio between CC₅₀ and IC₅₀ (SI = CC₅₀/IC₅₀).

Results and Discussion

Antioxidant Properties

Antioxidant defence system of cells comprises of endogenous antioxidants, such as superoxide dismutase, catalase, glutathione peroxidase, glutathione reductase, glutathione, ascorbic acid, uric acid, etc., which act either independently, or cooperatively, (or even synergistically) against free radicals (Vandana *et al.*, 2006). These antioxidants protect against the deleterious effects of reactive oxygen species by scavenging them, converting them to non-toxic compounds, or chelating the ions required for their activation. Cells suffer because of deterioration at physiological processes during oxidative stress, when the antioxidant defence system becomes inadequate to neutralize the excess reactive oxygen species (ROS) produced. The supplementation of exogenous antioxidants has been found to be effective in restoring the homeostatic disturbances due to oxidative stress. This supports the role of natural antioxidants in achieving strong immune system as well as healthy aging (Han *et al.*, 2005). This study has been held to explore the antioxidant effect of geraniol in pro-oxidant conditions, as compared with the reference standards ascorbic acid and butylated hydroxyl toluene.

The DPPH assay usually involves a hydrogen atom transfer reaction (Li *et al.* 2009). DPPH radical scavenging test is a sensitive antioxidant assay and depends on substrate polarity. The presence of multiple hydroxyl functions could be considered as an option for the hydrogen donation and/or radical scavenging activity.

Antiradical activities of tested substances against stable DPPH radical expressed in IC₅₀ [μg.L⁻¹] showed notable values (Table 1). A lower IC₅₀ value indicates a greater antioxidant activity.

Most active was BHT, the same activity demonstrated ascorbic acid, and least active was geraniol. As a rule, the antioxidant properties of the plant extracts cannot be attributed to activities of single constituents. Their scavenging activity could be explained by the combination of effects with one another. Ruberto and Baratta (1999) demonstrated that the most radical scavenging activities of natural extracts are mainly due to the cumulative effect of ingredients as polyphenols, as well as nerol, eugenol and geraniol; within their structure has been observed polar-bonded hydrogen. Undoubtedly, DPPH radical has little relevance to present in biological systems as well in living organisms, but this study is indicative of the capacity of geraniol to scavenge free radicals, and will refer to hydrogen atom or electron donation ability, independently of any enzymatic activity.

AOA of tested compounds in Fe²⁺/ascorbic acid-induced oxidation of egg liposomes are expressed as percentage of inhibition of oxidation process in comparison to control sample (without tested substances). Geraniol significantly depressed the effect of oxidation. It exhibited a protective capacity against Fe²⁺/ascorbic acid-induced lipid peroxidation in liposomes in a concentration-dependent manner.

The damaging reactions of free radicals are widely implicated in the etiology of numerous oxidative stress-related diseases (Piaru *et al.*, 2010). These typically electrophilic reactive moieties interact with lipids, proteins, and nucleic acids, and cause oxidative damages (Deighton *et al.*, 2010). Lipid peroxidation is one of the effects induced by free radicals, and it can occur in lipid system due to the presence of structures rich in highly peroxidizable, polyunsaturated fatty acids. The presence of antioxidants in the fraction will minimize the oxidation of these structures due to the inhibition of the chain reaction of lipid peroxidation (Sherry *et al.*, 2013). Antioxidant power of natural products is an expression of their capacity to defend from the action of free radicals as well as to prevent degeneration from oxidants (Deighton *et al.*, 2000, Piaru *et al.*, 2010, Sherry *et al.*, 2013).

Yu *et al.* (1995 a) reported that geraniol suppressed lipopolysaccharide-induced nitric oxide and prostaglandin E2 production at a system of RAW 264.7 macrophages in a dose-dependent manner. The inhibitory efficacy of geraniol was concomitant with decreases in protein and mRNA expression levels of inducible nitric oxide synthase (iNOS)

Although peroxidation in model membranes may be very different from peroxidation in cell membranes, the results obtained in the former membranes may be used to advance understanding of peroxidation in biological membranes (Schnitzer *et al.*, 2007).

Antiviral Test

The *in vitro* antimicrobial activity of eugenol against various pathogens has been reported earlier; very little is known about its activity and mode of action against viruses, which are important human pathogens assigned to different taxonomic groups: coxsackievirus B1 (CV-B1) from the *Picornaviridae* family, respiratory syncytial virus (RSV) from the *Paramyxoviridae* family, and influenza virus A/Aichi/68/H3N2 from the *Orthomyxoviridae* family. Therefore, the mode of antiviral action of eugenol against those viruses *in vitro* was evaluated in the present study. The results obtained demonstrated that geraniol was showing low cytotoxicity in a Hep2 cell system (Table 2). In the same model system, CC_{50} of geraniol is lower than that of disoxaril, used as reference substance for the study of antiviral effect of CVB1. Our research on antiviral screening of geraniol showed that there is no anti-

ral effect against influenza virus A/Aichi/68/H3N2, as well as against RSV. The scientific literature also lacks data published on this subject.

Pronounced antiviral effect was observed against the representative of the *Picornaviridae* family - coxsackievirus B1. IC_{50} of geraniol is 48 $\mu\text{g/mL}$, about 30% lower than oxoglaucone and about three times lower than disoxaril.

The antiviral activities of monoterpene alcohols (including linalool, nerol, citronellol, and geraniol) probably are due to their solubility in the phospholipid bilayer of cell membranes and increased permeability of cells (Knobloch *et al.*, 1989; Devi *et al.*, 2010).

These results suggest that geraniol exhibits anti-coxsackievirus B1 activity, supporting its therapeutic potential for virus-associated disorders.

In conclusion, geraniol is abundant and occurs in a large number of plants. This molecule is widely used as a fragrance chemical in both cosmetic and household products. Several studies have confirmed the pharmacological properties of this acyclic monoterpene alcohol. Geraniol - with its good chemopreventive activity - may present a new class of antiviral agent, and this renders a great opportunity for further investigation. The investigated biological properties of geraniol, including antiviral activities against some virus families, together with negligible toxicity, warrant further studies to explore the feasibility of formulating geraniol-containing consumer products with health promoting properties.

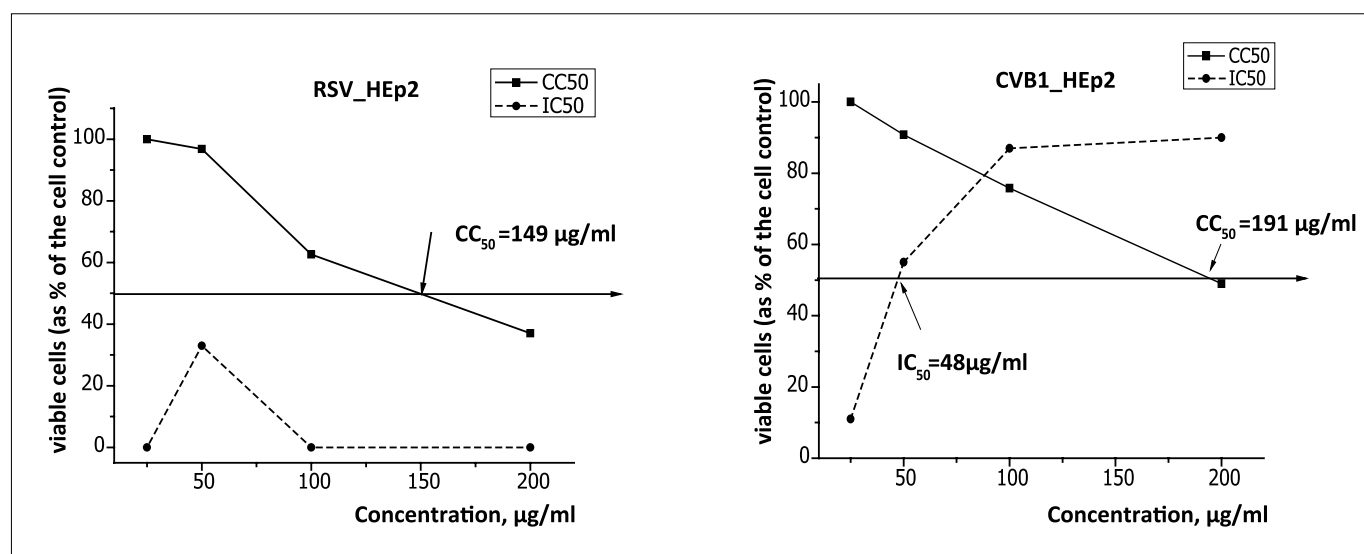


Fig. 2. Antiviral activity of geraniol against CVB1 and RSV virus in HEP2 cells. Data are present as CC_{50} – percentage viable HEP2 cells, and IC_{50} – percentage protection.

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Antibacterial Activity of Organically Grown Vegetables

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Abstract

In recent years many antimicrobial agents became ineffective against bacteria. This led to many studies of the antimicrobial effects among plants. Bulgarian vegetables are ingredients in many traditional Bulgarian foods. The aim of the study was to compare the antibacterial activity of organically grown parsley, tomatoes, cayenne peppers, onions and garlic against referent test bacteria - *Bacillus subtilis* No8751 and *Escherichia coli* No8752, supplied by the National Bank for Microorganisms and Cell Cultures. Sixteen vegetable samples were analyzed. Petri dishes were cultivated at 37°C for 24 hours. Our results demonstrated low antibacterial activity of tomato and parsley samples against the Gram(+) *B. subtilis* and from weak to no activity against the Gram (-) *E. coli*. Fresh garlic samples showed low antibacterial activity against Gram (+) bacteria with only fresh garlic leaves displaying wider sterile zones. No antibacterial activity was demonstrated by the fresh onion bulbs and leaves. In contrast, the mature garlic bulbs showed pronounced activity against the Gram (-) *E. coli* (30 mm zone d). Cayenne pepper tissue and seeds possess antibacterial properties against both *Escherichia coli* and *Bacillus subtilis*. Strong antimicrobial activity against *B. subtilis* was shown by the mature onion samples with 25 mm zone diameter for the red onion bulbs and 27 mm zone diameter for the white onion bulbs. Both mature onion samples demonstrated low activity towards *E. coli*.

Резюме

През годините много антимикробни вещества се превърнаха в неефективни. Това доведе до много нови проучвания за антимикробно действие сред растенията. Българските зеленчуци са съставки на много традиционни наши храни. Целта на проучването бе да се сравни антибактериалната активност на органично отглеждани магданоз, домати, лют червен пипер, лук и чесън срещу референтни тестови бактерии - *Bacillus subtilis* No8751 и *Escherichia coli* No8752, предоставени от Националната Банка за Микроорганизми и Клетъчни Култури. В проучването бяха използвани 16 растителни проби. Експериментите бяха проведени по метода дифузия в агар. Петритата бяха култивирани при 37°C в продължение на 24 часа. Нашите резултати показват ниска антибактериална активност на пробите от домати и магданоз срещу Грам (+) *B. subtilis* и от слаба до отсъстваща активност срещу Грам (-) *E. coli*. Пробите пресен чесън показаха ниска антибактериална активност срещу Грам (+) бактерии само листата от пресен чесън, показаха по-широки стерилни зони. Главите и листата от пресен лук не демонстрираха антибактериално действие. Зрелите глави чесън, обаче, показват ясно изразена активност срещу Грам (-) *E. coli* (30 мм диаметър на зони). Тъканите и семената от лют червен пипер притежават антибактериални свойства както срещу *E. coli*, така и срещу *B. subtilis*. Силна антимикробна активност срещу *B. subtilis* беше показана от пробите зрял лук с диаметър 25 мм при зоните от червен лук и зони с диаметър от 27 мм за главите бял лук. И двете проби зрял лук показаха ниска активност към *E. coli*.

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Introduction

Currently there is a need for new antimicrobial agents, because of the multidrug resistance of pathogenic bacteria. Many studies are focused on finding new bio-active compounds in extracts derived from plants. Preparations containing such compounds as the principal physiologically active constituents have been used to treat human diseases. Reports of activity in the field of antibacterial flavonoid research are widely conflicting, probably owing to inter- and intra-assay variation in susceptibility testing (Tim and Lamb, 2006). Yee-Lean *et al.* (2003) reports, that among the vegetable and fruit extracts, all fresh vegetables showed no antibacterial activity, but purple and red vegetable and fruit juices had antibacterial activities.

Petroselinum crispum has been widely used in folk medicine and has been reported to possess antibacterial activity against both Gram (+) and Gram (-) bacteria (Bin *et al.*, 2007; El Astal *et al.*, 2005; Peter *et al.*, 2006; Sayyednejad and Damab, 2008).

Tomatoes are potent antioxidants and scavengers of free radicals (Richard and Pajkovic, 2008). Tomato-based bio-films have been reported to protect against bacterial pathogens and spoilage while also enhancing sensory properties of foods (Wen-Xian *et al.*, 2008, 2012).

Capsicum species produce fruits that synthesize and accumulate unique hot compounds known as capsaicinoids in the tissues and seeds (Cesar *et al.*, 2011; Mariângela *et al.*, 2011). Bio-autographic tests have demonstrated that capsaicin is the main antimicrobial component (Soediro *et al.*, 1997). *Capsicum annuum* fruits have also been used in traditional medicine for their antibacterial activity against a broad spectrum of bacterial species (Mariângela *et al.*, 2011; Rose *et al.*, 2012; Soediro *et al.*, 1997). Antimicrobial, antifungal and antioxidant activity has also been reported for *Allium cepa*, showing it as a new potential source of bio-active compounds due to the presence of Flavonoids (Cammue *et al.*, 1995; Chun-Lin *et al.*, 2013;

Freddy *et al.*, 2006; Jonathan *et al.*, 2010).

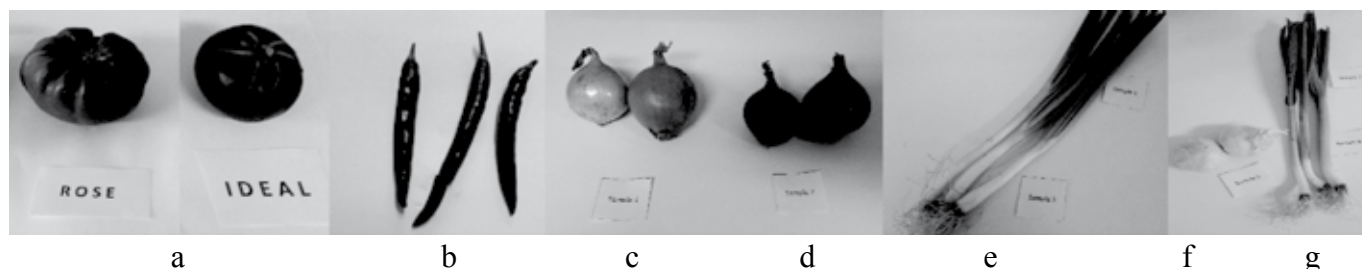
Garlic has been used for its medicinal properties for thousands of years, investigations on its mode of action were recently performed. Recent research shows *Allium sativum* as a promising source of antibacterial agents that can be used in food preservation and pharmaceuticals (Srinivasan *et al.*, 2009).

The geographic location of Bulgaria provides one of the best environments for cultivating organic vegetables rich in bio-active substances, without the need for chemically enriched manure or other soil additives. The aim of our study was to compare the antibacterial activity of organically grown Bulgarian parsley, tomatoes, peppers, onions and garlic against referent Gram (+) and Gram (-) test bacteria.

Materials and Methods

Nine different organically grown vegetables were used in our study: parsley, two varieties of tomato, cayenne peppers, white, red and fresh onions, mature and fresh garlic. The vegetable samples were divided in two parsley samples, eight tomato samples, three garlic samples and four onion samples. Samples were prepared in a way closest to human consumption. The samples were analyzed via agar diffusion method against Gram (-) *E. coli* and Gram (+) *B. subtilis*, supplied by the National Bank for Microorganisms and Cell Cultures.

- Parsley samples were separated in stems and leaves.
- Tomato samples from two varieties were used: D5 - "Rose" and D6 - "Ideal". The samples were prepared as dried tomato puree, raw tomato puree, tomato seeds and tissue slices in order to note the antibacterial properties of each part of the tomato fruit for both varieties.
- The pepper fruits were cut open with a sterile scalpel and were separated in 3 samples - raw tissues, tissue disks (d = 0.5mm) and seeds.
- The onion samples were divided in white and



Samples: tomatoes (a), cayenne peppers (b), white onion (c), red onion (d), fresh onion (e), mature garlic (f) and fresh garlic (g)

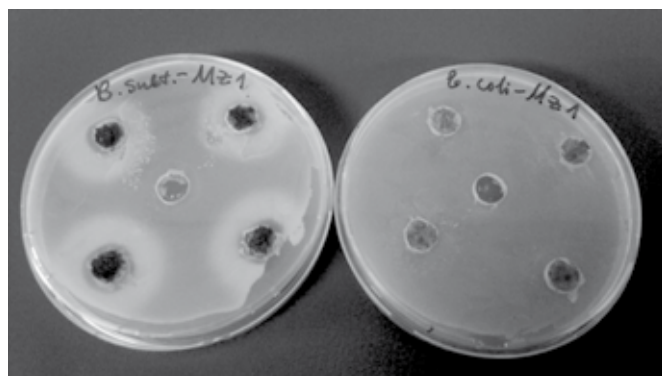


Fig. 1. Antibacterial activity of parsley leaves against *B. subtilis* N°8752 and *E. coli* No8751

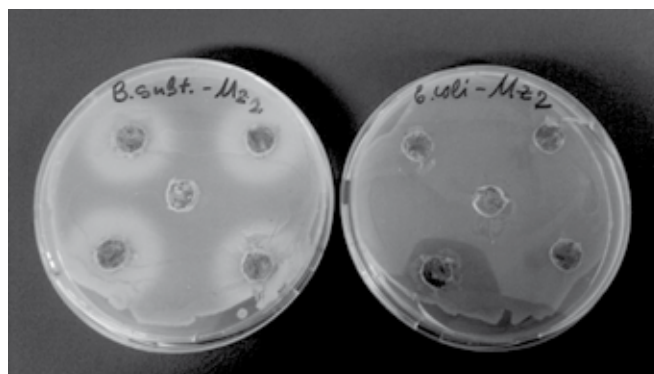


Fig. 2. Antibacterial activity of parsley stems against *B. subtilis* No8752 and *E. coli* No8751

red onion bulbs, fresh onion bulbs and fresh onion leaves.

- Mature garlic bulbs, fresh garlic bulbs and fresh garlic leaves were used for the garlic samples.

The parsley leafs and stems were homogenized using a mortar and pestle, while the tomato, onion and garlic samples were separately homogenized using a sterilized blender. The dried tomato puree was prepared by drying part of the raw puree at 37°C for 24 hours. The cayenne pepper tissues were scraped away with a sterile scalpel.

Nutrient agar petri dishes were inoculated with 0.1ml 1×10^9 CFU/ml of the test microorganisms the Gram (-) *E. coli* No8752 and Gram(+) *B. subtilis* No8751 (supplied by the National Bank for Microorganisms and Cell Cultures). Wells (d = 9mm) were then aseptically perforated in the Nutrient agar to hold the homogenized samples and the raw cayenne pepper tissues.

The tomato and pepper seeds, tomato tissue slices and the pepper tissue disks were placed on the surface of the inoculated agar.

Petri dishes were cultivated at 37°C for 24 hours. Sterile zones around the wells, seeds and slices were then measured.

Results and Discussion

The obtained results demonstrated diverse effects of the vegetable samples on the test bacteria's growth ranging from no to strong antibacterial activity as follows:

Parsley Antibacterial Activity

Parsley leafs inhibit the growth of *B. subtilis* (2 mm inhibition zone) and are not active against *E. coli*. Similar results were obtained for parsley stems: no antibacterial activity was registered (Fig. 1, Fig. 2). At the same time, El Astal *et al.* (2005) reports that *E. coli* was more affected by the ethanolic parsley extracts than Gram (+) bacteria (6).

Raw Tomato Puree Antibacterial Activity

Wan-Xian D.'s research (2008) shows that edible films made from tomatoes containing carvacrol possess antimicrobial effects against *E. coli*. Our investigation demonstrated that the raw puree, from sample D5 ("Rose") exhibited no antibacterial activity against *B. subtilis* and *E. coli*. Sample D6 ("Ideal") displayed antibacterial activity against both *B. subtilis* and *E. coli* with zone diameter of 4 mm against *B. subtilis* and about 4.5 mm against *E. coli*. (Fig. 3)

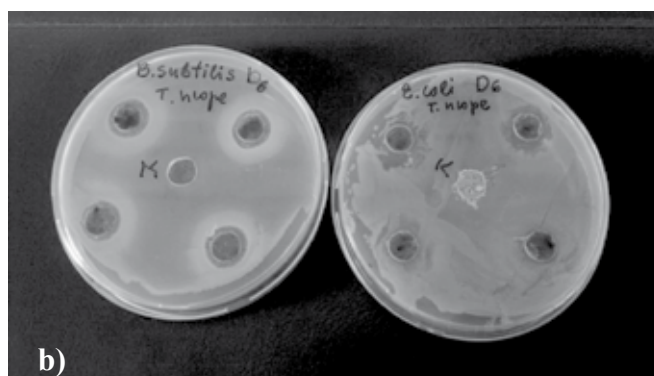
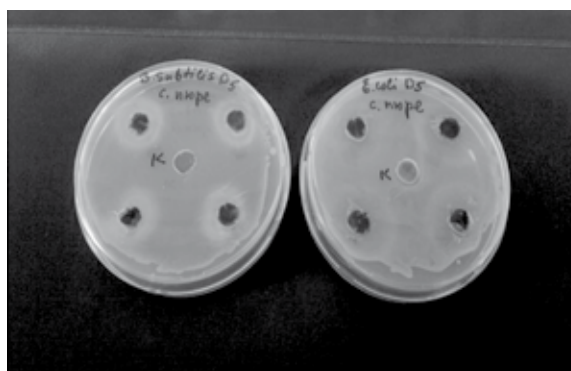
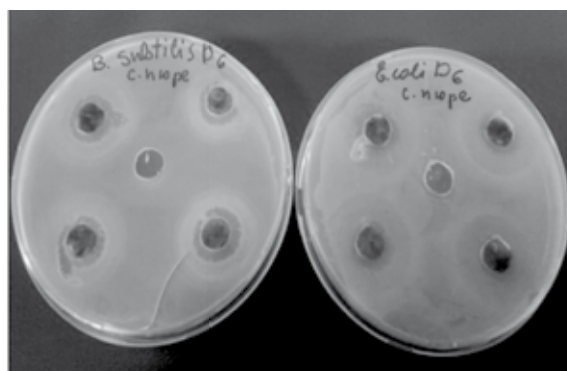


Fig. 3. Antibacterial activity of raw tomato puree, of variety D5 (a.) and D6 (b.), against *B. subtilis* N°8752 and *E. coli* N°8751.



a)

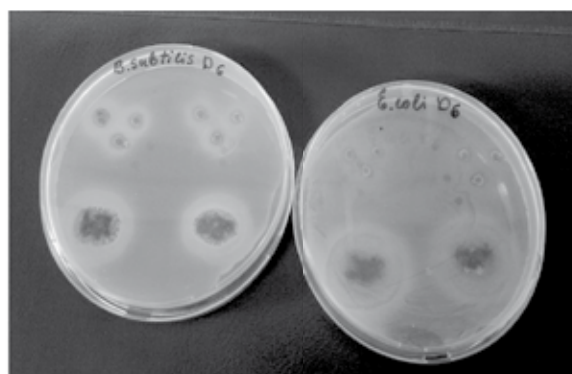


b)

Fig. 4. Antibacterial activity of dried tomato puree, of variety D5 (a.) and D6 (b.), against *B. subtilis* N°8752 and *E. coli* N°8751.



a)



b)

Fig. 5. Antibacterial activity of tomato seeds and tissue slices, of variety D5 (a.) and D6 (b.), against *B. subtilis* N°8752 and *E. coli* N°8751.

The D5 dried puree samples also displayed no antibacterial activity against *B. subtilis* and *E. coli*. The D6 dried puree samples also displayed larger sterile zones than the D5 dried puree samples against both bacteria - with zone diameter of 5 mm against *B. subtilis* and about 3.5 mm against *E. coli* (Fig. 4).

Tomato tissue slices from samples D5 and D6 display no antibacterial properties against *B. subtilis*, or against *E. coli*. (Fig. 5)

Tomato seeds of samples D5 (Rose) and D6 (Ideal) inhibit the growth of *B. subtilis*, but display no antibacterial activity against *E. coli*, for both samples D5 and D6. The D5 ("Rose" variety) sample exhibited sterile zones with range between 4 mm. and 7 mm, while the D6 ("Ideal" variety) zones had a range of about 5 mm. A more recent research made by Naseer *et al.*, (2012) showed results similar to ours as it reports low antibacterial activity in tomato fruits against *E. coli* (Fig. 5).

Cayenne Pepper Antibacterial Activity

Cayenne pepper fruits and seeds inhibit the growth of both *E. coli* and *B. subtilis*. Pepper tissues displayed no antibacterial activity. Cayenne pepper

discs showed antibacterial activity against both *E. coli* (25 mm inhibition zone diameter) and *B. subtilis* (24 mm). The seeds also exhibited inhibition zones of 11 mm against *E. coli* and 7 mm against *B. subtilis*. The results for the pepper tissues are not shown (Fig. 6 and Fig. 7). Other recent and previous studies, like the ones conducted by Mariângela *et al.* (2011) and Soediro *et al.* (1997), also report the Capsicum's fruit antibacterial activity against both Gram (+) and Gram (-) bacteria.

Onion Antibacterial Activity

The authors Cammue *et al.*, (1995) and Jonathan *et al.*, (2010) describe *Allium* extracts to be more effective against Gram (+) microorganisms, while Gram (-) bacteria were reported to be less susceptible. Freddy *et al.* (2006) described water extracts from yellow onion skin to be active against Gram (-) bacteria. All of our onion samples showed antibacterial activity against both Gr (+) and Gr (-) bacteria except the fresh onion bulbs. White and red onion bulbs displayed inhibition zones of 27 mm and 25 mm against *B. subtilis* and 3 mm against *E. coli*. (Fig. 8). Fresh onion bulbs and leaves showed no antibacterial activity against both bacteria strains (Fig. 9).

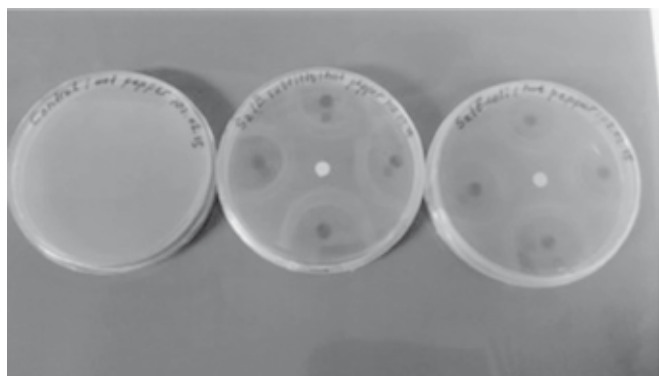


Fig. 6. Antibacterial activity of Cayenne pepper tissue discs against *B. subtilis* N°8752 and *E. coli* N°8751.

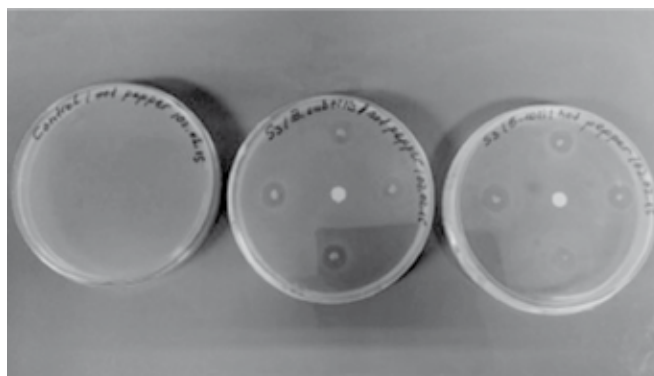
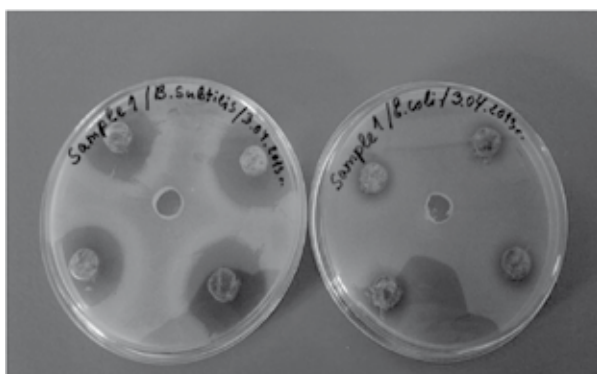
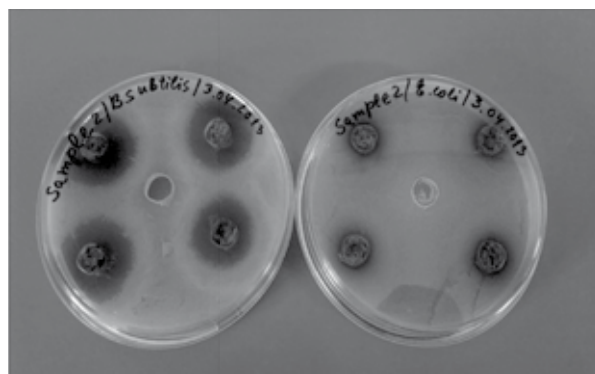


Fig. 7. Antibacterial activity of Cayenne pepper seeds against *B. subtilis* N°8752 and *E. coli* N°8751.



a)



b)

Fig. 8. Antibacterial activity of white (a.) and red (b.) onion bulbs against *B. subtilis* N°8752 and *E. coli* N°8751.

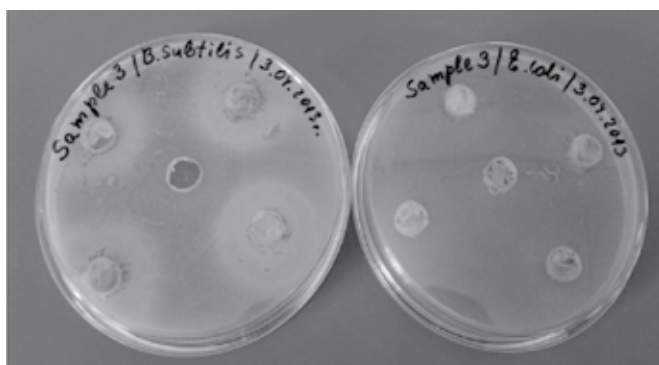
Garlic Antibacterial Activity

Mature and fresh garlic bulbs demonstrated low antibacterial activity against *B. subtilis* (7 mm for mature garlic bulbs and 2 mm for fresh garlic bulbs). Fresh garlic bulbs displayed no activity against *E. coli*, but mature garlic bulbs showed inhibition zones on 30 mm against *E. coli*. (Fig. 10) The fresh garlic leaves displayed antibacterial activity against both *E. coli* (13 mm) and *B. subtilis* (17 mm). Srinivasan *et al.*, (2009) reports antibacterial

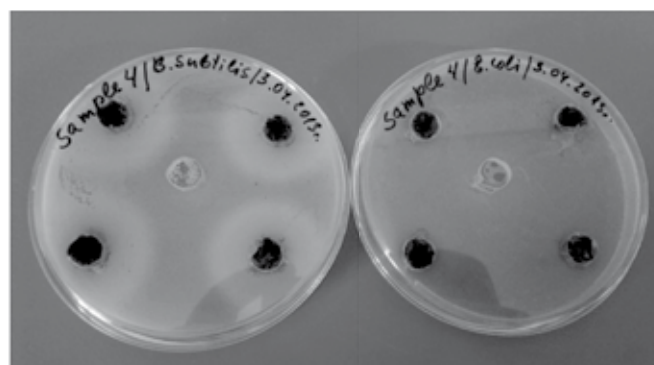
activity of *Allium sativum* against both Gram (+) and two Gram (-) pathogenic bacteria, with the maximum zone of inhibition observed against *Bacillus subtilis*. His analysis revealed that the antimicrobial effectiveness is time- and temperature-dependent (Fig. 11).

Conclusions

In conclusion, cayenne peppers showed antibacterial activity against both *B. subtilis* and



a)



b)

Fig. 9. Antibacterial activity of fresh onion bulbs and leaves against *B. subtilis* N°8752 and *E. coli* N°8751.

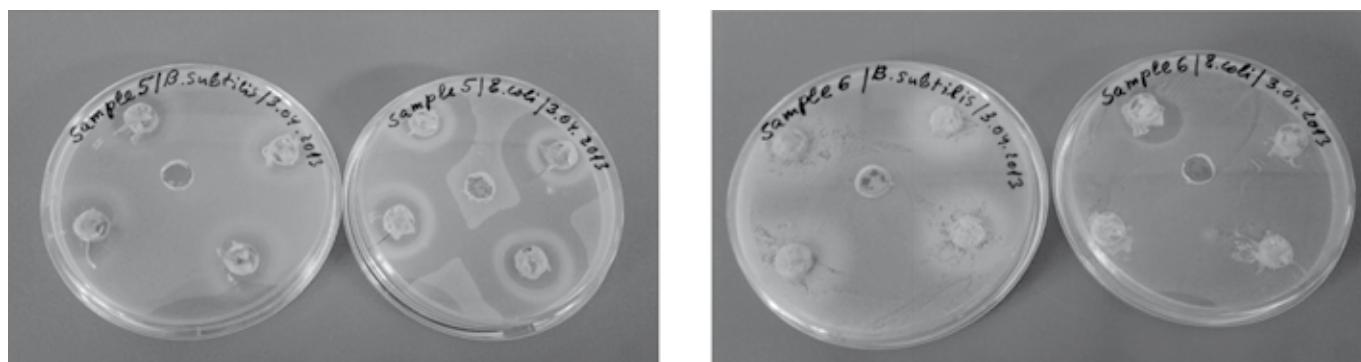


Fig. 10. Antibacterial activity of mature (a.) and fresh (b.) garlic bulbs against *B. subtilis* N°8752 and *E. coli* N°8751.

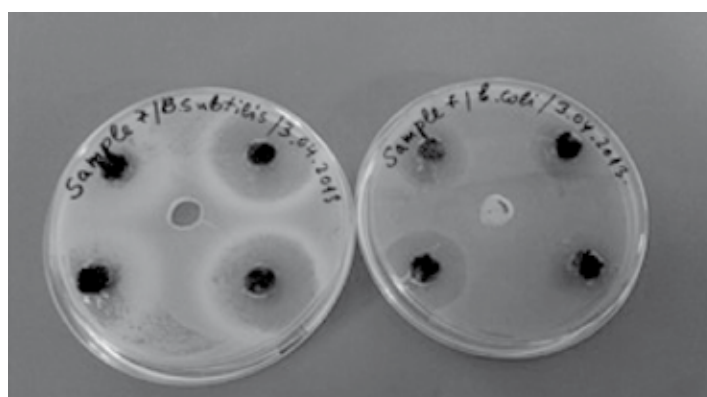


Fig. 11. Antibacterial activity of fresh garlic leaves against *B. subtilis* N°8752 and *E. coli* N°8751.

E. coli. Mature onion bulbs were active against only *B. subtilis*. Greatest antibacterial activity was demonstrated by mature garlic bulbs. Moderate antibacterial activity was observed by fresh garlic leaves. Our results demonstrated low antibacterial activity of tomato and parsley samples against the *B. subtilis* and from weak to

no activity against the *E. coli*.

Acknowledgements

The study was conducted at the Bio Laboratory of New Bulgarian University and was supported by the Faculty of Basic Education of New Bulgarian University.

Table 1. Antibacterial activity of organically grown vegetables

Sample	Parsley		Tomato								Cayenne Pepper			Onion				Garlic		
	Parsley stems	Parsley leaves	Raw puree		Dried puree		Seeds		Tissue Slices		Raw tissue	Tissue discs	Seeds	White Bulbs	Red Bulbs	Fresh bulbs	Fresh leaves	Mature bulbs	Fresh bulbs	Fresh leaves
			D5	D6	D5	D6	D5	D6	D5	D6										
<i>B. subtilis</i>	0	2	0	4	0	5	6	5	0	0	n.a.	24	7	27	25	0	0	7	2	17
<i>E. coli</i>	0	0	0	4.5	0	3.5	0	0	0	0	n.a.	25	11	3	3	0	0	30	0	13

All figures are in mm

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A Complex Theoretical Approach for Algal Medium Optimization for CO₂ Fixation from Flue Gas

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Abstract

An robust algorithm of medium optimization for culturing algae was developed. The algorithm takes into account elemental composition of algae, and chemical composition of non-organic source of carbon (in our case, flue gas). Values of the chemical elements in the medium is achieved in linear programming procedure. Medium composition may change on the base of the chosen objective function, for example biomass maximization or overproduction of desired metabolite. The algorithm was checked by performing real experiments with *Chlorella vulgaris* and *Scenedesmus* species in our previous studies (in USA). The results have shown a very good algae growth, when the medium was optimized by using such algorithm. The developed robust algorithm for medium optimization is under continuous verification (in Bulgaria and Brazil) on different algae cultivation processes. The algorithm can be successfully used to design a medium for any bacterial and algae cells with known or unknown physiological requirements for macro- and micro-elements.

Key words: medium optimization, linear programming, algae, flue gas, elemental composition of cells.

Резюме

Разработен е устойчив алгоритъм за оптимизация на хранителна среда за култивиране на водорасли. Алгоритъмът отчита елементния състав на водораслите и химичния състав на неорганичния източник на въглерод (в нашия случай, димни газове). Стойностите на химичните елементи в средата се изчисляват с помощта на метода на линейно програмиране. Съставът на хранителната среда може да се променя на базата на избрания критерий за оптимизация, за максимизиране например биомаса или свръхпродукция на желан метаболит. Алгоритъмът е проверен чрез извършване на реални експерименти с щамове на *Chlorella vulgaris* и *Scenedesmus* в предишни наши изследвания (в САЩ). Получените резултати показват много добър растеж на водораслите, когато средата се оптимизира чрез използване на този алгоритъм. Проверката на разработения устойчив алгоритъм за оптимизация на хранителна среда се извършва непрекъснато (в България и Бразилия) върху различни процеси на култивиране на водорасли. Алгоритъмът може успешно да се използва за изчисляване на компонентите на хранителната среда за всякакви бактерии и водораслови клетки с известни или неизвестни физиологични изисквания за макро- и микроеlementи.

Introduction

Medium optimization for algae culturing is a key point in overall process development. As a common practice, medium optimization for biomass and high value products is done by using statistical experimental design methods (Kathiresan *et al.*, 2007) or simple try and error procedure. Because CO₂ represents a considerable part of the

operational costs in mass production of algae (Kadam, 1997) a flue gas can be considered as an excellent choice. In order to reduce the transportation costs, a flue gas from a local coal-fired plant must be used as a source of carbon dioxide for culturing photosynthetic algae. From another point of view, the cultivation of microalgae can also be taken into account as an additional step in flue gas treatment, where CO₂ concentration in the exhaust flue gas is decreased and SO₂ emissions causing acid rain

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can be totally eliminated. Global warming situation accelerates political decisions and many countries directed much attention and financial support on studies of the subject.

Hence, utilization of flue gas from coal-fired power plants (Maeda *et al.*, 1995a; Kadam, 2002), industrial heater burning kerosene (Chae *et al.*, 2006), natural gas-fired boiler (Doucha *et al.*, 2005; Doucha and Livansky, 2006), or a simulated flue gas (Brown, 1996) were performed in attempt to make CO₂ fixation from flue gas (Benemann, 1993) a visible cost-effective industrial scale technology. Special attention in the field must be given to international achievements in Europe, Israel, Japan and USA.

However, these studies do not focus on the complex algorithm and how the first steps in process development (medium optimization) were performed. The studies showed a global solutions and ranges of applicability of this technology without showing any details about optimal values of medium components and how they were obtained. As well, there are no studies giving understanding on interactions between water chemistry-algal physiology-flue gas.

Following this thoughts we tried to build a robust algorithm for medium optimization by applying the principles of system analysis theory (Kaffarov *et al.*, 1979, 1985), modern mathematical methods and available software for calculation of chemical equilibrium and speciation of CO₂ and SO₂ in water in order to understand such interactions. The algorithm was used in a step-by-step pro-

cedure of active experimental design by culturing *Scenedesmus* an *Chlorella* species with the goal of optimal inorganic carbon utilization from flue gas emissions. The main idea of this robust method was presented in several meetings of Society of American Engineers in Massachusetts and elsewhere in USA (Crofcheck *et al.*, 2009a, 2009b, 2010, 2012a) and as well as elsewhere (Kroumov, 2013; 2014; Kroumov *et al.*, 2014). The experimental verification of this approach can be found in details elsewhere (Crofcheck *et al.*, 2012b).

In this work, we are going to highlight the theoretical steps of the algorithm and how this knowledge can be used in other levels of complex photobioreactor (PBR) model development. Experimental verification of the developed algorithm was successfully performed at Biosystem Agricultural Engineering Lab, and CAER Lab, at University of Kentucky under the grant “CO₂ sequestration from flue gas by algae for production of lipids, carbohydrates and proteins”.

First, several medium recipes for culturing fresh water *Chlorella* were analyzed by applying knowledge on elemental composition of algae biomass and linear programming calculations.

Secondly, N-source, P-source, and micro-elements composition were changed and varied in order to design the cost-effective medium for closed PBR in outdoor cultivation conditions. The flue gas is rich with metals which must be considered as micro-nutrients. Finally, we tried to simulate the SO₂(aq) influence on the microalgae growth and to understand SO₂(aq) interactions with algae for the

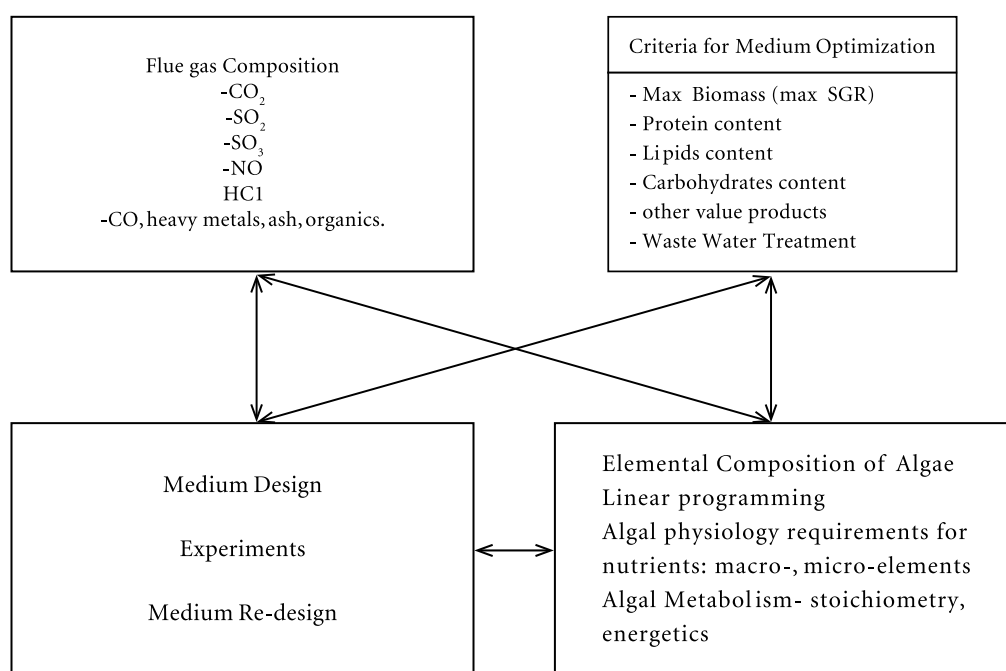


Fig. 1. Graphical representation of the complex theoretical approach for medium optimization

given pH. Water chemistry studies during absorption of flue gas showed that pH may reach values of pH=2.0 which requires buffering the medium or precise pH control. Buffering the medium with sodium bicarbonate as a best choice was studied, as well.

Theory-Development of the Algorithm

Existence of very rich knowledge database on fresh and marine algae physiology and their elemental composition, metabolism, and nutrients requirements for fastest growth opens the space for synthesis of optimal algorithms for medium optimization. Hence, development and application of chemically defined medium is not a single act, it is a procedure which should be focused on practical considerations, especially process economics and the complex interactions of the medium with the environment (absorption of flue gas composition in water, its water chemistry which is linked with the algae physiology). It means interactions between simple chemical components with algae and aqueous species of flue gases (mainly obtained from dissolution of CO₂, O₂, SO₂, SO₃, HCl, NO) must be understood. Development of defined medium and its industrial application should relay on reproducible results. The simplicity of the medium by considering all available resources of macro- and micro- nutrients (from water and flue gas) should be a criterion, as well, in order to permit unlimited long term cultivation. Studying and describing such a system cannot be done in a straightforward procedure and requires decomposition of the system in sub-systems and their analysis and synthesis. Hence, for precise calculation of medium components, an application of software packages (for example, MAPLE) with modern numerical and optimization methods (linear programming) is necessary. Studying water chemistry requires knowledge about thermodynamic properties of chemical components and their possible interactions. MINEQL+4.6 software was used as a powerful tool for studying and evaluating chemical equilibrium aqueous speciation of gases, solid phase saturation states, and precipitation-dissolution.

Combining this knowledge database, we developed an algorithm for medium optimization which is based on the principles of system analysis theory (Fig. 1) (Kaffarov *et al.*, 1979, 1985). The sub-systems were determined as follows-I-algae, II-flue gas, III-water chemistry. Understanding of these sub-systems and their interactions pass through formulation of the optimization criterion

and “procedure of making of decisions” (medium design-experiments-medium re-design).

I - Algae physiology can be considered as a main sub-system. The theory was based on evaluation of the fresh water medium for culturing *Chlorella* strains. The physiological requirements of fresh water algae can be taken from the elemental composition of the particular strain. Nevertheless, evaluation of the published chemical composition of *Chlorella* is fundamental for algorithm development. The strain and medium selection for industrial application is a loop procedure and here is not going to be discussed.

Algae Biomass Elemental Composition

The mass balance strategy is well known one from chemical engineering practice. Applying it to algal biotechnology is very useful and robust tool when there is no obvious starting point for medium design or selection of available recipes for the given algae strain. We performed the analysis on inorganic medium components which present into the biomass, where the biomass nitrogen content was the reference point.

We assume for simplicity that elemental composition of *Chlorella* sp. does not differ significantly; hence for all calculations, one algae elemental composition was used (where “max” values of chemical elements were chosen.). As first step in medium development, we evaluated the most common medium recipes (such as -N-8 and M-8 (Mandalam, and Palsson, 1988), BG-11 (Borowitzka and Borowitzka, 1988), M4N medium (Watanabe and Saiki, 1997), Watanabe Medium (Scragg *et al.*, 2002), used in practice for mass culturing of fresh water algae. The criterion of unlimited growth was achieved by exact calculation of the medium components. We applied linear programming technique (coded in MAPLE 12) based on elemental composition of *Chlorella* specie biomass to calculate the exact amount of chemical components and to compare the simulation results with the real recipe values. *Note: Elemental composition values (Min, Max) for N, P, K, Mg, S, Fe, Zn, Cu, Mn were obtained from Oh-Hama and Miyachi, 1988, Chlorella, p. 3-26, in Micro-algal biotechnology, Michael A., and Lesley J. Borowitzka, Cambridge University Press, 1988, p.477.*

For our calculations, the most important 9 inorganic macro-and micro-elements (N, P, K, Mg, S, Fe, Zn, Cu, Mn) were considered. For all simulations, the maximum (Max) values of the chosen algal biomass elements were considered.

Linear Programming-Approach and Application

From the average elemental composition of biological material 9 elements were selected that occur in the highest concentrations. Of course the remaining trace elements can also become depleted, but they are only required in very small quantities, which are supposed already to be present in sufficient amounts as impurities in the used chemicals. A number of inorganic chemical compounds can yield the required elements either as free ions or as small ionic molecules that can readily be absorbed. Development of defined synthetic medium is based on perfect proportion between chemical elements representing stoichiometric elemental composition of biomass. To calculate the required quantities for the various chemicals the method of linear programming, more particularly the algorithm for search of a constrained maximum was used.

The program for linear programming calculations was coded in MAPLE 12 software (property of University of Kentucky, Department of Mathematics). In linear programming, a vector “x” is found that minimizes the quantity $c \cdot x$ subject to the constraints $m \cdot x \geq b$ and $x \geq 0$, in which the vector “x” represents the required quantities of the various chemicals, “c” represents the variable to be minimized (e.g., the number of chemicals to be used or the costs of the chemicals per unit), “b” gives the required concentration in the mixture. The composition of the various chemicals may include one chemical element, which should be considered during the calculations. More flexibility can be achieved when the constraints for each variable can be varied. The algorithm constraints search for and find the global maximum of a function in the domain specified by a number of equations (equalities or strict or non-strict inequalities). It must be noted that algorithm is not restricted to the use of linear programming as a maximum search method. For algal medium design can be used statistical (factorial design) and modern search methods such as - genetic algorithms, artificial neural networks, fuzzy sets and their combinations.

Algae Physiology Requirements for Nutrients

The maximum cell density and specific growth rate of algae as a function of nutrients and environmental conditions are well documented in books (Borowitzka and Borowitzka, 1988; Amos, 2004). This knowledge directed our scientific efforts toward the study of optimal nitrogen sources including fertilizers for *Chlorella* maximum

growth. For flue gas applications it is important to evaluate the influence of micro-elements such as V, Mo, Co, Ni and utilization of yeast extract in some applications. From financial point of view, it is challenging to evaluate the *Chlorella* growth response on completely fertilizer medium with combination of wastewaters rich with inorganic nutrients. In this sense, water recycling can be considered as an option, as well but some physiological test on auto-toxins accumulation and influence on overall process performance must be performed.

Algal metabolism interacts with the environment by changing its pH value. It is challenging to determine the ability of algae to synthesize low molecular compounds siderophores (Naito *et al.*, 2008) in order to utilize the chemical components of the medium which precipitates (Fe, Ca, Mg ions) under the high pH values $pH > 8.0$ and become bio-unavailable.

Algae Metabolism

General approach for medium design is based on solely empirical data and the cells are considered as a black box system. Of course, achievements in metabolic engineering showed a new direction in medium design strategies. This direction is to evaluate the metabolic pathway needs of the algae cells. Combination of stoichiometric models of intracellular metabolites and the mass balance approach seems to have bright future for the medium design. In the future, detailed metabolic and energy flux analysis is expected to be of tremendous help in medium development procedure. Current approaches of medium design in biotechnology includes utilization of the statistical factorial design methods (Kim *et al.*, 2008; Jeong *et al.*, 2008) and the most modern optimization techniques artificial neural networks (Kennedy, 1992), fuzzy sets (Kennedy *et al.*, 1995), genetic algorithms (Weuster-Botz *et al.*, 1995; Zuzek *et al.*, 1996) and their combinations, but once again many experiments are required and the cell is considered as a black box. It must be noted, that application of statistical and optimization techniques is very powerful tool for medium design, but they are not “panacea”. Their search space must be localized between algal metabolic and financial constraints. Further developments on this subject are expected.

II-Flue Gas as a Sub-System

According to our algorithm of medium design, two important interactions of the flue gas in the liquid phase can be considered.

1. pH changes of the medium during absorption of flue gas.
2. How the flue gases speciation in the liquid phase will affect the algae growth.

Flue gas composition and absorption in aqueous and alkaline solutions is well studied and modeled process in chemical engineering practice (Marocco and Inzoli, 2009; Gomez *et al.*, 2007; Desch, *et al.*, 2006). From these studies is obvious that pH of the non-buffered liquid phase dramatically changed and can reached pH=1-2. Hence, buffering the broth for performing algae non-limiting growth on flue gas is obligatory and NaOH (NaHCO₃) is the best choice from chemical (Wylock *et al.*, 2008; Chang and Rochelle, 1985), and micro-algal physiology point of view (Hsueh *et al.*, 2007). To catch CO₂ from flue gas by chemical reaction to produce carbonates (e.g., Na₂CO₃) and use the latter as the carbon source for micro-algal cultivation is very attractive perspective.

For some algae species, combination of SO_x and NO_x have some toxic influence on the algae growth (Lee *et al.*, 2002).

Hence, special interest has been given on algae cultivation process by using flue gas (actual or simulated, where algae tolerance to high CO₂ concentration in presence of SO_x and NO_x impurities was extensively studied (Maeda *et al.*, 1995b; Yanagi *et al.*, 1995; Negoro *et al.*, 1991, 1993; Lee *et al.*, 2002; Jeong *et al.*, 2003).

The flue gas compositions varies from one to another coal-fired plant and its content can be found in the literature. We focused our attention on CO₂ (up to 15%) and SO₂ (up to 700 ppm) content which were crucially important to build the proper and optimal medium.

III Water Chemistry

Analyzing the flue gas composition the influence of SO_x can be considered crucially important. We assume that the problems with ash, organics and heavy metals presented in flue gases have been solved and only gases are entering PBR.

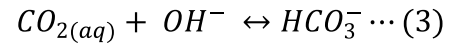
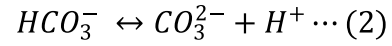
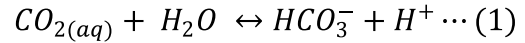
Hence, aqueous phase chemistry of the flue gases must be completely understood in order to be applied effective unit operation for gases absorption and their latter utilization by algae.

Evaluation of flue gas composition showed that we may safely restricted our analysis to the dynamics and instantaneous equilibrium reactions of six dissolved species namely: CO₂(aq), HCO₃⁻; CO₃²⁻, SO₂(aq), HSO₃⁻; SO₃²⁻. Oxidation of SO₃(g) in the gas and liquid phases gives sulfate which is

a component of algal medium. All other gases can be considered as no harmful impurities and their interactions with algae and chemical species in the liquid phase will be neglected.

Water Chemistry of Flue Gas (CO₂ and SO₂)

The absorption of flue gas in water and alkaline solutions is well studied physical-chemical process and details can be found elsewhere. For our practical purposes special interest is given to the process of flue gas absorption into bicarbonate/carbonate solution (Ebrahimi *et al.*, 2003). The authors modeled speciation of CO₂ and SO₂ flue gas components by considering the following chemical reactions

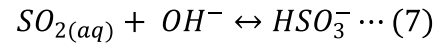
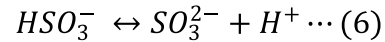
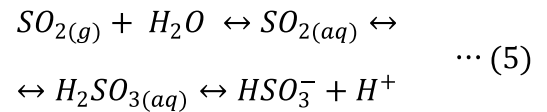


$$[C_{Total}] = [CO_{2(aq)}] + [HCO_3^-] + [CO_3^{2-}] \dots (4)$$

where C_{total} - stands for total dissolved inorganic carbon dioxide concentration.

Water Chemistry of SO₂

Dissociation of SO₂ includes the following reactions



$$[SO_{Total}] = [SO_{2(aq)}] + [HSO_3^-] + [SO_3^{2-}] \dots (8)$$

where SO_{total} -stands for total sulfite concentration.

According to them the reaction (2, 5 and 6) are very fast. Hence, instantaneous equilibrium is assumed for the reactions (2), (5) and (6) throughout the liquid phase. The hydrolysis of CO₂ is slow. Two reactions (1) and (3) take place in the absorption process of CO₂ by aqueous alkaline solutions. Details about rate and dissociation constants values

and their temperature dependence are cited by the authors accurately. It can be noted that the forward rate constants values of reactions (1) and (3) differ (in orders) as a function of pH values below 8 and above 10. Thus, buffering the algal medium with sodium bicarbonate (as a depot of CO₂) or pH control by NaOH has to be considered.

Procedure of Making of Decisions

“Medium design - Experiments - Medium re-design” we called sub-system where the decisions are made on the base of all available information from other sub-systems for the chosen criterion of optimization. It must be noted that the “pure” water chemistry, “pure” flue gas absorption and speciation, and “pure” algae growth can be evaluated (on the base of literature data) separately without experimental verification. Only, interactions between

sub-systems and concurrent hypothesis should be checking out for particular working conditions and algae species.

Criterion for Medium Optimization

Determination of criterion for medium optimization is different in research studies as well as in the studies on industrial applications. Criterion for research studies usually is selected to maximize the algae growth under high CO₂ content in the liquid phase. The medium may contain different sources of growth factors and vitamins supporting maximum growth. On the other hand, CO₂ sequestration in industrial conditions requires minimization of costs; hence the medium must be optimized by taking into account algal physiology and growth of algae on cheapest possible sources of nitrogen, phosphorus etc. It means available wastewater source rich with

Table 1. Evaluation of N-8 and M-8 medium composition on the basis of elemental composition of the biomass and by applying linear programming procedure (LPP)

Medium				
Chemical compound	N-8	LPP results	M-8	LPP results
-	g/L	g/l	g/L	g/L
Macro-elements of the medium				
KNO ₃	1.0	1.000	3.0	3.000
KH ₂ PO ₄	0.74	0.158	0.74	0.474
NaHPO ₄	0.26	0	0.26	0
CaCl ₂ .2H ₂ O	0.013	0.00528	0.013	0.00158
FeSO ₄ .7H ₂ O	0.00001	0.0493	0.13	0.148
MgSO ₄ .7H ₂ O	0.05	0.146	0.4	0.437
Micro-elements of the medium				
	g/L	g/l	g/l	g/L
MnCl ₂ .4H ₂ O	0.01298	0.000648	0.01298	0.001944
CuSO ₄ .5H ₂ O	0.00183	0.000283	0.00183	0.000848
ZnSO ₄ .7H ₂ O	0.0032	0.000396	0.0032	0.00119

inorganic component can be an option.

Designing the medium under different criteria makes possible to prepare catalog of defined synthetic media which will serve in pilot plant studies and further their industrial applications in closed PBR configurations. This approach is predetermined because constraints applied for designing the medium for lab studies and industrial design are different. Most common constraints and challenges encountered in lab studies are as follows: time for medium development; cost of development efforts; lack of shaker space; precipitation reaction; foaming; water quality; dispersion or dissolution of solid medium components; effect of components on assay techniques; effect of components on downstream product purification.

For design of an industrial medium most common encountered constraints are as follows: availability of raw materials throughout the year; batch to batch variability of medium components; transport costs of medium components; medium cost and price fluctuations of medium components; contamination problems; water recovery of used medium.

Following this algorithm, *Chlorella vulgaris* UTEX 2714 growth behavior and *Scenedesmus* one was studied in different media and conditions. The experimental verification of the method can be found elsewhere (Crocheck *et al.*, 2012).

First Step

As a first step in this algorithm we have focused on evaluation of the media compositions most commonly used for mass culturing of *Chlorella* sp. We chose the maximum values of 9 components (N, P, K, Mg, S, Fe, Zn, Cu, Mn) in order to calculate the defined medium by avoiding any algae growth limitations. Further, we evaluated the most common medium recipes (such as -N-8 and M-8 (Mandalam and Palsson, 1988), BG-11 (Borowitzka and Borowitzka, 1988), Watanabe's Medium (Scragg *et al.*, 2002) and M4N medium (Watanabe and Saiki, 1997), used in practice for mass culturing of *Chlorella*).

For lab studies in flasks BG-11, M4N, and Watanabe's Medium were eliminated. However, their recipes can be re-designed and applied for different purposes in outdoor big scale, when vigorous mixing and high light intensity conditions during the summer time are common.

Evaluation of N-8 and M-8 medium was done previously by authors (Mandalam and Palsson, 1998). The authors' criterion was biomass maxi-

mization (high density culture) and their conclusions were that the N-8 medium is deficient in iron, magnesium, sulfur, and nitrogen at high cell densities and long term cultivation. It should be noted that no difference in biomass and chlorophyll content of *Chlorella vulgaris* was observed during 120 h of cultivation in controlled bioreactor conditions.

By using elemental composition of biomass and linear programming procedure we evaluated in what extend these two medium were stoichiometrically balanced. The results are shown in Table 1. The assumption of performing LPP procedure were as follows: CO₂ in the liquid phase, light availability, and mixing are not limiting factors; N-source is reference for calculation of biomass concentration; sodium ions are not limiting for fresh water algae and are not included in LPP because in bioreactor conditions these ions are supplied by both tap water and titration in controlling pH level.

The software program of LPP was coded in MAPLE 12. The LPP results have shown that one can expect biomass concentration of 1.799 g/L for N-8 medium and 5.398 g/L for M-8 medium, respectively in controlled bioreactor working conditions. Comparing the medium recipes with LLP results the following conclusion can be made:

First, phosphates concentration in both medium is high. Addition of NaHPO₄ can be avoided. Calcium concentrations difference is not crucial for real outdoor cultivation of fresh water algae because tap water or industrial water is a source of Ca. Sulfates are not a problem, as well, which is in agreement with water chemistry of flue gases SO₂, SO₃. Magnesium, and iron in N-8 medium are not optimal, which was shown by the authors (Mandalam and Palsson, 1998). In our case, micro-elements of both medium are not a concern because flue gas and technical grade chemical components are their good source.

Hence, the algorithm works well and without performing the experiments we were able to build a kinetic hypothesis and to localize the optimum area of experimental study. Moreover, the very first experiment with *Chlorella vulgaris* UTEX 2714 in flasks showed an excellent result.

For preliminary study, we choose M-8 medium for flask experiments to evaluate the growth behavior of 3 algae strains. *Chlorella vulgaris* UTEX 2714 and *Chlorella sorokiniana* UTEX 1230 were obtained from the culture Collection of Algae at The University of Texas at Austin, USA. A wild "unidentified strain" was isolated from water near the Eastern Kentucky Coal-Fired plant (data are

property of U-ty of Kentucky and are not shown). The algorithm works excellent and can be applied for development of any synthetic fresh and sea water medium.

Also, it must be noted that the best medium chosen from flasks experiments not obligatory will be the best medium for large scale cultivation in PBRs. The problem is that during the scale-up procedure, the medium composition is changed to meet the control strategies requirements, for example, fed batch addition of nutrients.

According to our algorithm of medium development emphases can be given on following experiments by using fresh water algae and flue gas as a non-organic carbon source.

1. Experiments with M-8 medium -results and discussion;
2. Experiments with urea -results and discussion;
3. Experiments with different medium components and deionized and tap water- results and discussion;
4. Experiments with micro-nutrients -V, Co, Mo, Ni- results and discussion;
5. Experiments without complexation of Iron by EDTA to study siderophores synthesis ability in algae (Naito et al., 2008)- results and discussion;
6. Experiments -buffering the system-Titration with H_2SO_4 and buffer $NaHCO_3$.
7. Experiments with simulated flue gas (Na_2SO_3)- results and discussion;

Note: Based on the LPP, many important medium with industrial application industrial for culturing algae were evaluated. (For example: BG-11 medium; Zarrouk's Medium; Bold's Basal Medium-BBM; Ogbonna-Tanaka Medium; Modified Ogbonna-Tanaka Medium). We have build a catalog of media recipes and the LPP verification is under way through the Brazilian project "Science without borders", 2014-2017.

Conclusions

This work demonstrated that a complex algorithm for medium optimization for culturing algae (in our case *Chlorella*) can be successfully applied in lab conditions by minimizing money, time and scientific efforts. The chosen strains performed well with urea as a nitrogen source. Microelements were necessary for maximum growth of *Chlorella* and the strain utilized yeast extract as a source of vitamins. The findings well complemented most of the previous reports, which focused mainly on

culturing *Chlorella* on flue gas. Analysis of the maximum specific growth rate (SGR) showed high potential of the strain for CO_2 sequestration from flue gas. Fertilizer medium can be safely used as a substitution of M-8 synthetic medium for outdoor mass production.

The main advantage of the algorithm is that avoids excessive unproductive experimentation. Its flexibility allows selecting the strains and medium in optimal way (loop procedure) before scale up to outdoor conditions in order to prepare different scenarios of micro-algal performance and adaptability.

In order to achieve very high productivity of such alga, medium re-design and different mode of controlled operations must be studied in pilot plant scale where light availability, mixing conditions and process control strategies are going to play crucial role.

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Short Communication

Antibacterial activity of *Lactobacillus bulgaricus* G-LB-44

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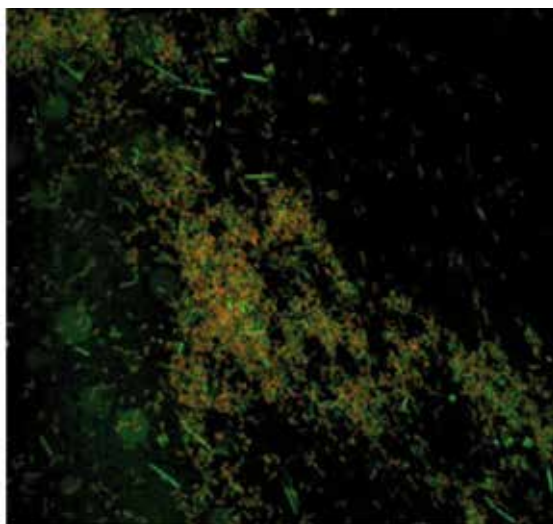
Lactobacillus bulgaricus G-LB-44 was screened for antibacterial activity against (i) different pathogenic species - *Escherichia coli*, *Listeria monocytogenes*, *Staphylococcus aureus*, *Acinetobacter baumannii*, *Enterococcus faecalis*, *Pseudomonas aeruginosa* and (ii) 16 lactic acid bacteria strains and 2 commercially available probiotics using plate counting. All counts were recorded as colony-forming unit per millilitre (CFU/mL). *Lactobacillus bulgaricus* inhibition of *H. pylori* has been initially manifested by Boyanova et al. (2009).

The results showed that *Lactobacillus delbrueckii* subsp. *bulgaricus* G-LB-44 could reduce the growth of potentially harmful bacteria that cause diseases in humans. In most cases, the reduction in the numbers of pathogenic bacteria was greater than 99%. The broad based activity of *Lac-*

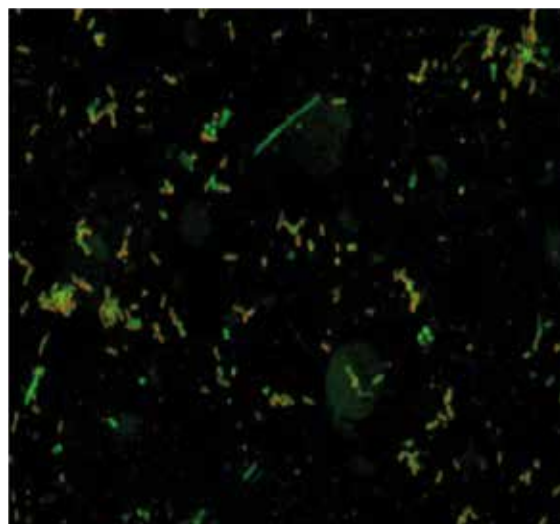
tobacillus delbrueckii subsp. *bulgaricus* G-LB-44 is unusual among *Lactobacillus* species and suggests that there are unique cellular components that may account for this activity. Such compounds are common in nature, however they tend to be directed to very specific strains of other bacteria that compete for nutrients with the probiotic bacteria in a natural setting such as the gastrointestinal tract. It is, therefore, unusual to find a single strain of *Lactobacillus* that produces an inhibitory effect for a broad array of harmful bacteria, since these bacteria would rarely be found at the same time within the same natural setting. Moreover, with regard to other lactic acid bacteria strains and commercial probiotics, no inhibitory activity of *Lactobacillus delbrueckii* subsp. *bulgaricus* G-LB-44 was observed. The study confirms that G-LB-44 is a specific strain

Table 1. Pathogen inhibition by *L. bulgaricus* G-LB-44

Organism	Strain	Inhibition rate; [%]
<i>Acinetobacter baumannii</i>	ATCC 19606	>99
<i>Enterococcus faecalis</i>	ATCC 29200	>99
<i>Enterococcus faecalis</i>	ATCC 29212	>99
<i>Enterococcus faecium</i>	ATCC 51559	>95
<i>Escherichia coli</i>	ATCC 25922	100
<i>Escherichia coli</i>	ATCC 35150	>99
<i>Listeria monocytogenes</i>	ATCC BAA-751	100
<i>Listeria monocytogenes</i>	Clinical Isolate	100
<i>Pseudomonas aeruginosa</i>	ATCC 27853	100
<i>Salmonella typhimurium</i>	Clinical isolate	100
<i>Shigella sonnei</i>	ATCC 325931	100
<i>Staphylococcus aureus</i>	ATCC 12600	>99
<i>Staphylococcus aureus</i>	ATCC 25923	>99
<i>Staphylococcus aureus</i>	ATCC 29213	>98



3 hrs



24 hrs

Fig. 1. Comparison of the whole biofilm structure and the cell density at different time points of the test

of the *L. bulgaricus* and that its inhibitory power against pathogens surpasses other *L. bulgaricus* strains. G-LB-44 is an example of an effective probiotic with suitable scientific substantiation of health benefits.

Lactobacillus bulgaricus G-LB-44 is able to significantly decrease the cell density of *E. coli* biofilm as well as to destroy completely the *E. coli* biofilm structure within a 24 hour period as demonstrated in Figure 1, in images obtained by confocal laser scanning microscopy.

E. coli biofilms growing on glass slides were incubated with *L. bulgaricus* G-LB-44 for 3 and 24 hrs. The bacteria populations were stained with Live/Dead viability kit (Invitrogen) at the end of the incubation period, following the manufacturer's instructions. Fluorescence microscopy images were acquired by a Leica CLSM TCS-SP confocal microscope with a 63.0 X lense. Green short cells: live *E. coli*; red short cells: dead *E. coli*; green long cells: live *L. bulgaricus* G-LB-44.

Since the sub-species of *Lactobacillus bulgaricus* has been safely used in foods for over 100 years with no indications of overdose or side effects, we

believe that the above results indicate the potential of this strain to be used both as a natural preservative capable of inhibiting pathogens in unpasteurized food and as away to replenish the healthy bacteria found in the human digestive system.

Acknowledgements

The authors thank Prof. Angel S. Galabov, MD, DSc for the special contribution and guidance during this study, Genesis Laboratories for providing their proprietary strain of *L. bulgaricus* G-LB-44 for the purpose of this study, the Section of Morphology of Microorganisms and Electron Microscopy of the Stephan Angeloff Institute of Microbiology, Bulgarian Academy of Sciences for the preparation of the biofilm samples and the Light Microscopy unit of the Hellenic Pasteur Institute, Athens, Greece for the production of the microscopy images.

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Letter to the Editor**Clinical Experience with *Lactobacillus bulgaricus* GLB44 in *Helicobacter pylori* (+) Patients**

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Introduction

The purpose of this study is an assessment of the effect of *Lactobacillus delbrueckii* subsp. *bulgaricus* G-LB-44 in *Helicobacter pylori* (+) patients.

Methods

The monitoring included twenty-four patients at the average age of $45,46 \pm 13,3$ years, of which 50% were women. All patients were *Helicobacter pylori* positive (+). The infection was evident by rapid urease test (RUT), fecal antigen test, a breath test, and histological examination, or by a combination of these methods. Unsuccessful eradication therapy was conducted in six of the patients in the past and the rest of them have not been treated previously. Esophagogastroduodenoscopy was performed in all patients with the following findings: 26.1% had gastroesophageal reflux disease, 65.2% - hiatal hernia, 87% - gastric changes, 4.3% - duodenal erosions, and 21.7% - active duodenal ulcer. Enrolled course conducted by administration of *Lactobacillus delbrueckii* subsp. *bulgaricus* G-LB-44 (capsules and tablets) at a daily dose of 15×10^9 in combination with Rabeprazole 2×20 mg or Pantoprazole 2×20 mg for seven days followed by *Lactobacillus delbrueckii* subsp. *bulgaricus* G-LB-44 individually for three days at the same dosage (15×10^9). In all patients was carried out the control fecal antigen test for *Helicobacter pylori* after at least 43 days post treatment.

Results

In 22 patients (91.7%) the control fecal antigen test was negative for *Helicobacter pylori*. In two patients (8.3%) the control study showed persistent *Helicobacter pylori* infection. Both patients belonged to the group of previously treated patients, who have previously failed eradication with different antimicrobial drugs. The remaining four patients of the group of the previously treated patients (one of them was with autoimmune gastritis) had negative control *Helicobacter pylori* test. Patients did not manifest adverse reactions or side effects when taking *Lactobacillus delbrueckii* subsp. *bulgaricus* G-LB-44 (ProViotic®).

Conclusion

This preliminary human trial demonstrated a novel effective method of treating patients with *Helicobacter pylori* (+) infection without the use of antibiotics.

In memoriam



Andreas Engibarov
1929 - 2015

The eminent microbiologist, mycobacteriologist, and immunologist, the respected colleague and most loved friend Senior Research Associate Dr. Andreas Engibarov has departed from this world. We ask forgiveness from a worthy and highly erudite man, who has left a bright trace in the Bulgarian microbiology and immunology.

The pages of his countless favorite books (probably the most favorite one from his childhood was "Peter Pan") remain not completely read; the thousands records from his personal collection with the preferred works of Bach, Händel, Mahler, Wagner (with his favorite symphonic poem "Thus Said Zarathustra" by Richard Strauss) remain not listened to the end...; the conversations, lighted up by his everlasting laughter, remain uncompleted...

A scientist with unusual inquisitiveness to nature, medicine, history, literature, music, who concentrated his scientific interests on the mycobacteria and mycobacterioses, and on the most recent and less examined issues of the specific immunological prophylaxis of tuberculosis.

Dr. Andreas Engibarov was born in Varna on May 2, 1929. Among the enates in his family were: Prof. Dr. Georgi Pavlov (1881-1945), who gradu-

ated veterinary medicine in Turin and worked in Dobrudzha (Bulgaria) - one of the founders and a chairman of the Union of Scientific Workers; Academician Stefan Pavlov (1914-1993) - a notable theoretician of the Bulgarian criminal procedural law. Andreas' childhood passed among books and music. When he was 10-year-old, his family moved to Sofia, where he graduated the First Male Secondary School in 1947. After graduating in medicine in 1954, according to his own wish he went to practice in Dobrudzha (the villages of Lovchantsi, Vladimirovo, and Karapelit) as a district doctor. He succeeded in obtaining an old radiographic apparatus from Ruse for the Radiographic Office of the small District Hospital in the village of Karapelit. He also founded a Clinical Laboratory. He appeared at a contest at the National Institute of Epidemiology and Microbiology (National Center of Infectious and Parasitic Diseases) in 1958. Still a student he worked as a demonstrator at the Department of Microbiology - he had lost his father when being a first-year man and needed to earn his living. A few were those who believed that a peasant doctor could win this consent. However, he succeeded! He specialized in the USA for a year - in the Harvard University in Boston and in the University of California in Los Angeles. After being chosen for Senior Research Associate, he became Head of the Laboratory for Specific Prophylaxis of Tuberculosis. The high quality of the Bulgarian Bacillus Calmette-Guérin (BCG) vaccine, which is exported in more than 150 countries worldwide today, is one of the greatest achievements of the National Center of Infectious and Parasitic Diseases and the Bulgarian microbiology.

The results of the studies of Dr. Engibarov on the discovery of new methods for microbiological diagnostics of infections, caused by Mycobacterium tuberculosis, as well as on the taxonomy of the mycobacteria, represented a valuable contribution for the Bulgarian mycobacteriology. In the 70s of the XXth century Dr. Engibarov made the beginning of the species identification of the atypical mycobacteria in Bulgaria, based on the classical and modern microbiological and biochemical methods. A great practical value had the epidemiological studies on infections, caused by atypical mycobacteria. Parallel with the intensive studies on

perfecting the methods of production of tuberculin preparations (together with Senior Research Associate Dr. E. Sapundzhieva), he performed studies on the properties of tuberculin active protein. His attainments in the study of tuberculin active peptides achieved wide international acknowledgment. A special interest represented the results of the joint studies with Senior Research Associate V. Kolushki on the antigens of BCG and on virulent mycobacteria. The introduction of production of purified protein derivate (PPD) of tuberculin was a major achievement of the applied microbiology. The efforts of the team, led by Dr. Engibarov, for improvement of the quality and standartization of PPD of tuberculin were permanent, and they culminated with complete success. The standartization of the skin tuberculin test had major significance for the effectiveness of the specific prophylaxis of tuberculosis.

Dr. A. Engibarov was an initiator of joint international studies on the standartization of the anti tuberculosis BCG vaccine. He worked with enthusiasm on improvement of the methods for control of vaccine quality. The new methods, developed by him, for characterization of the BCG vaccine (one of which was the fast test for measuring of the intracellular content of ATP as a method alternative to the cultural for determination of the viability) currently are an object of joint international studies, organized by the World Health Organization. Still in the 80s of the last century, Dr. Engibarov and Dr. Stoyan Bardarov, using highly efficient methods of molecular biology, laid the foundation of a new generation of anti tuberculosis vaccines. Today, 30 years later, the challenges and progress of this vaccinal strategy are widely discussed. A consent is achieved regarding the turning to a clinical assessment of the most promising “candidates” for new vaccines (modified BCG vaccine or attenuated *Mycobacterium tuberculosis*) (Tom Ottenhoff, Stefan Kaufman (2012); Vaccines against Tuberculosis: Where Are We and Where Do We Need to Go? *PLoS Pathog* 8(5):e1002607). However, the BCG vaccine is still the only one used and the only effective vaccine in the treatment of tuberculosis (and it will remain the only one in the coming years).

Dr. Engibarov had a huge contribution to the development of a BCG vaccine especially designed for immunotherapy of malignant neoplasms - Calgevax. Under his management were performed detailed experimental, pathomorphological, immunological, and biochemical stud-

ies. Upon representing the first results of clinical studies, Dr. Engibarov met scepticism in some of the members of the Scientific Council. However his ambitiousness and tenacity overcame this difficulty. Many countries show interest of this preparation today. It is applied in all types of malignant tumors, when adjuvant immunotherapy is required. Especially wide is its intravesical application in patients with superficial tumors of the urinary bladder. The observations of many years by leading oncologists (the first one was Senior Research Associate Dr. R. Ikonopisov) in patients with malignant melanoma showed the favorable results from the use of Calgevax as an agent for adjuvant immunotherapy.

Dr. Engibarov was an author of more than 180 scientific publications - articles and reviews - in Bulgarian and foreign scientific issues. He managed scientific sessions of a series of scientific forums in Bulgaria and abroad. He actively participated in the organization of the scientific life of the Scientific Society of Epidemiology, Microbiology, and Immunology, as well as of the Society of Microbiology at the Union of Scientific Workers. He was a scientist with a critical disposition towards the problems in the development of science and university education. His erudition and brilliant foreign language knowledge were amazing. The successes in the work of the Science and Information Center at the National Center of Infectious and Parasitic Diseases would never be achieved without his everyday tenacious contribution in the work of the associates of the Center.

After his retirement he participated actively in the public life. He was a municipal counsior - and a Secretary of the Metropolitan Municipal Council for a short period of time - during the hard way towards democracy in the 90s. He was a deservedly respected, honorable member of the Bulgarian Association of Microbiology.

I have asked myself many times whether he had taken that very manifested dignity and joy, that he emitted, from the thousands books he had read? He will remain for these qualities in our hearts for ever. Because “for the man is the joy, the joy and the laughter, that he has to learn”, as Friedrich Nietzsche wrote in “Thus Said Zarathustra”.

Let us honor with a deep, deep bow the bright memory of Dr. Andreas Engibarov!

Milyana Chuchkova

Chronicle

Second National Scientific Food Conference with International Participation, March 20-21, 2015, Sofia

Currently, the food safety is a topic of mankind's great interest. In March 2015 at New Bulgarian University the IInd National Scientific Food Conference with international participation was held. The conference was organized by the Bulgarian Society for Microbiology, New Bulgarian University (NBU), Department of Natural Sciences and Biolaboratory, The Stephan Angeloff Institute of Microbiology, Bulgarian Academy of Sciences, and the Bulgarian Food Safety Agency. The Organizing and Scientific Committee members were prominent scientists of Bulgarian Academy of Sciences, Sofia University, University of Food Technologies in Plovdiv and top-level managers of the Bulgarian Food Safety Agency. The Second Food Conference was under the auspices of New Bulgarian University's Rector. It was financially supported by the Central Fund for Strategic Development of NBU as well as by the laboratory equipment business companies. Recent results of the NBU Biolaboratory and the ongoing projects laid the foundations of the Food Conference and its future organization in each two consecutive years.

During the conference the 110th Anniversary of the discovery of *Lactobacillus bulgaricus* by Dr. Sta-

men Grigoroff was celebrated. A solemn speech was given by Academician Angel Galabov.

The conference was a large-scale local scientific event gathering together over 150 delegates who presented 100 plenary lectures, oral presentations and posters in six topics: Food Quality, Food Biotechnology, Food Safety, Microbiological Control of Food, Probiotics and Prebiotics and Varia. Great interest was paid on the plenary lecture of Prof. Vaso Taleski (University "Goce Delchev", Stip, FYROM) focused on the nanomicrobiology, the application of nanobiosensors in detecting food pathogens and the advantages of metal nanomaterials that possess unique antimicrobial activities as silver, gold and titanium. Prof. Encho Savov (Military Medical Academy, Sofia, Bulgaria) gave a key plenary lecture that explored the end of the post-antibiotic era and multidrug- and pandrug-resistant bacteria. Assoc. Prof. Milen Georgiev (Stephan Angeloff Institute of Microbiology, BAS) presented a plenary lecture on metabolomics applied in natural products and biotechnology. A novel probiotic product was presented by Kiril Petkov. The product contains *Lactobacillus bulgaricus*, which was isolated from mountain snowdrop.



It is currently patented in USA and on the USA market. The product inhibits in 48h 100% of the growth of pathogenes *Escherichia coli*, *Listeria monocytogenes*, *Staphylococcus aureus*, *Acinetobacter baumannii* as well as *Pseudomonas aeruginosa*.

The conference attended both Bulgarian and foreign scientists from Switzerland and Balkan Peninsula countries - Serbia, FYROM and Kosovo.

The conference coursed as a small congress with plenary lectures, oral presentations and a poster session with more than 60 posters. Three posters were selected by the Scientific Committee for The Best Poster of Young Scientist Award: 1st place was awarded to the poster of Zahmanov *et al.* (Bulgaria) called "Beyond the nutritional value of *Sambucus ebulus*: metabolomics and antioxidant activities"; 2nd place won the poster of Nemska *et al.* (Bulgaria) on the topic "Isolation and initial characterization of lactic acid bacteria from traditional dairy products". The 3rd place was honored to Logonia *et al.* (Serbia) for their research "Electrochemical determination of redox potential in infant formula

and human breast milk". All awarded authors were PhD students.

The second day of the Conference was devoted to a Workshop on Molecular Methods in Food Control. The Workshop was conducted in the Biolaboratory of New Bulgarian University, with the participation of ELTA-90, BIORAD and Progene Molecular Diagnostic Ltd.

More information is available at the conference website: <http://ebox.nbu.bg/2foodconference/>. Abstract book can be downloaded be the above mentioned website. Presentations and posters were included and edited in a CD-ROM, ISBN 978-954-535-624-7.

The next, Third International Food Conference will be held at March 23-25, 2017, in New Bulgarian University, Sofia.

Galina Sachanska
New Bulgarian University, Biolaboratory



General Information

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Registration fees

Early registration fee until June 20, 2015 - 150 €
Late registration fee after June 20, 2015 - 200 €
Registration fee on site - 250 €
Early registration fee for young specialists (up to 35 years old) until June 20, 2015 - 90 €
Late registration fee for young specialists (up to 35 years old) after June 20, 2015 - 130 €
Registration fee for young specialists (up to 35 years old) on site - 150 €

Registration fee includes:

- Access to the plenary sessions
- Access to the company presentations
- Access to the laboratory and microbiology equipment exhibition
- Access to poster sessions
- The material of the Congress
- Welcome Reception on October 22, 2015
- Coffee breaks
- Does not include accommodation

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Acta Microbiologica Bulgarica

GUIDE FOR AUTHORS

The journal *Acta Microbiologica Bulgarica* is organ of the Bulgarian Society for Microbiology (Union of Scientists in Bulgaria). The journal is continuation of the edited till 1993 journal of the same name, cited in Index Medicus and Medline. The new edition is published two times per year.

Types of articles

The journal publishes editorials, original research works, research reports, reviews, short communications, letters to the editor, historical notes, etc from all areas of microbiology. The manuscripts should not represent research results which the authors have already published or submitted in other books or journals. The papers submitted for publication in *Acta Microbiologica Bulgarica* are peer-reviewed by two experts from the respective scientific field who remain anonymous to the authors.

Article structure

The papers are published in English, accompanied by a bilingual summary (English and Bulgarian). The papers should be typed with double-spacing (28-30 lines), on a white paper in A4 format, with margins of 3 cm.

The length of the original research paper, including the annexes (tables, figures, etc.) should not exceed a signature or printer's sheet (30,000 signs, that is, 16 pages with 30 lines each) in Times New Roman 12. The length of shorter reports should not exceed seven pages. The submitted manuscript must contain the name/s and surname/s of the author/s, the name and address of the institution and or organisation where it was prepared. The name and address of the corresponding author should be noted. The abstract should not exceed 250 words and should represent briefly the goals, methods, main results (with numerical data) and basic conclusions of the research. The most essential six key words must be added to the abstract. The manuscript contains the following sections: introduction, materials and methods, results, discussion, acknowledgements, references. The introduction must be concise with a clearly defined goal and with previous knowledge of the problem. The materials and methods ought to contain sufficient data to enable the reader to repeat the investigation without seeking additional information. The results should be presented briefly and clearly, and the discussion should explain the results. The measuring units and other technical data should be given according to the SI-system. Illustrations (tables and figures) are submitted separately and their places in the text should be clearly indicated. Tables should be given in a separate sheet, numbered and above-entitled. The figures must be accompanied by legends in a separate sheet.

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