

Review**Oxidative Stress as a Target for Medication of Influenza Virus Infection**

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Abstract

During influenza pandemics in the last few years many people have died from severe complications associated with this pandemic despite receiving intensive care. This suggests that a definitive medical treatment for severe influenza-associated complications has not yet been established. About the pathogenicity of flu, many studies have shown that superoxide anion produced by macrophages infiltrated into the virus-infected organs is implicated in the development of severe influenza-associated complications. Selected antioxidants, such as alpha-tocopherol, N-acetyl-L-cysteine, glutathione, ascorbic acid, 5,7,4-trihydroxy-8-methoxyflavone, catechins, quercetin 3-rhamnoside, iso-quercetin etc. inhibit the proliferation of influenza virus and scavenge superoxide anions. The combination of antioxidants with antiviral drugs synergistically reduces the lethal effects of influenza virus infections. These results suggest that the combination of an agent with antiviral activities and an antioxidant could be a drug of choice for the treatment of patients with severe influenza-associated complications. Hopefully, this update of knowledge of antioxidant therapy of flu could be used as a potential approach to overcome the influenza-associated complications.

Key words: influenza virus infection, oxidative stress, antioxidants, specific inhibitors, combination therapy

Резюме

По време на грипна пандемия в последните години много хора са починали от тежки усложнения, въпреки получаване на интензивни грижи. Това предполага, че сериозна стратегия за медицинско лечение на тежко протичащия грип, все още не е установена. Много изследвания са показали, че супероксид анион произведени от макрофаги проникнали в инфектираните органи е свързан с развитието на тежки грипни усложнения. Някои антиоксиданти като α -токоферол, N-ацетил-L-цистеин, глутатион, аскорбинова киселина, 5,7,4-трихидрокси-8-метоксифлавон, катехини, кверцетин 3-рамнозид, изо-кверцетин др. инхибират пролиферацията на грипния вирус и улавят супероксидните аниони. Комбинацията от антиоксиданти със специфични антивирусни лекарства синергично намалява пагубните ефекти на грипните вирусни инфекции. Тези резултати предполагат, че комбинацията на специфичен антивирусен препарат и антиоксидант може да е успешна стратегия за лечение на пациенти с тежки усложнения, възникнали след грипна инфекция. Актуализирането на знанията за такива комбинации при профилактика и терапия на грип могат да бъдат потенциален подход за преодоляване на грип-свързаните усложнения.

Introduction

Influenza virus infection is a most widely spread illness and a major public health problem usually occurring in the northern hemisphere between the months of December and April. Epidemics of influenza are characterized by an increased morbidity and mortality in the community. Each year, seasonal influenza epidemics are responsible for 3–5 million cases of severe illness and 200 000–500 000 deaths worldwide. For this reason the

prevention and treatment of influenza infections is of particular importance (Vlahos, 2012).

Influenza virus A is a member of the *Orthomyxoviridae* family of enveloped, segmented, negative-strand RNA viruses. This virus replicates in the epithelial cells lining the upper respiratory tract of humans, and in both the upper and lower respiratory tract of mice (Ghoshal *et al.*, 2001).

One of life's paradoxes is the fact that the

molecule supporting aerobic life –oxygen - is not just essential for energetic metabolism and for respiration, but almost equally involved in the ethio-pathogenesis of numerous diseases and degenerative states due to oxygen-based reactive species called free radicals. Nature has selected and included, in an evolutionary manner, in the composition of living bodies, reactions generating free radicals with multiple roles: functional, intercellular communicational or destructive, cytolytic, etc. Free radicals occur in the body as the result of endogenous metabolic activity or of the local assimilation of some chemical pollutants at cellular level or at the level of several tissues, simultaneously or gradually. Due to their high reactivity, free radicals have been found responsible for many noxious effects on the living body. Oxidative stress is defined as an exaggerated production of oxygenated free radicals, accompanied by a dislocation of antioxidative agents. We cannot live without oxygen, since it is essential in the functioning of energy-producing cells. A body transforms and eliminates oxygen (as CO₂) properly almost entirely (98%). Free radicals produced by one's own body play a role in the cell defense system, destroying bacteria and viruses, decomposing chemical pollutants, and neutralizing toxins (Peterhans, 1997a,b; Ghoshal *et al.*, 2001; Vlahos, 2012).

The purpose of this work is to analyze the role of reactive oxygen species and development of oxidative stress in the pathogenesis of influenza viral infection, as an area of target for medication of flu. Attention should be focused on: (i) the effect of the virus on activation of phagocytic cells to release of free radical generation and pro-oxidant cytokines such as tumor necrosis factor; (ii) the effect of the virus on the pro-/antioxidant balance in host cells; (iii) effects of the redox state of the cell on the genetic composition of the virus as well as ROS-mediated release of host cell nuclear transcription factor-kappa-B, resulting in increased viral replication; and (iiii) efficacy of antioxidants as therapeutic agents administered individually and in combination with specific inhibitors of viral replication (Peterhans, 1997a; Peterhans, 1997b; Vlahos, 2012).

Oxidative stress as a key of influenza virus pathogenesis

The influenza infection starts with virus infusion in the lungs (Fig. 1). A cascade of processes follows:

- Massive infiltration of the infected tissue by

leukocytes, mainly PMNs, in the alveolar space, after the inoculation of influenza virus;

- Decrease in the partial pressure of oxygen and the development of hypoxia;
- Increase in the partial pressure of CO₂ and development of metabolic acidosis;
- Release of cytokines and prostaglandin E₂, and an enhanced immune response;
- Reducing the levels of endogenous lipo- and water-soluble antioxidants, as well as compensatory changes in the activity of antioxidant enzymes, massive bronchitis and pneumonia (Peterhans, 1997a,b; Mileva *et al.*, 2000, 2002a,b).

This concept of viral pathogenesis was put to the test using a mouse model of influenza (Hennet *et al.*, 1992; Peterhans, 1997; Lui *et al.*, 2003). Animals infected intranasally develop severe systemic symptoms, including a decrease in body temperature and weight, and anorexia at days five and seven post infection. The infection remains restricted to the airways and lungs, but the symptoms involve the whole infected organism. Viruses are parasitic pathogens that replicate in living cells, exploiting multiple intracellular pathways in their hosts for their own advantage and growth. As a rule, viruses cause a wide array of inflammatory and neurological diseases, leading to serious consequences (Fig. 2). Both viral products and host mediators can cause tissue damage, and depending on the cell type and

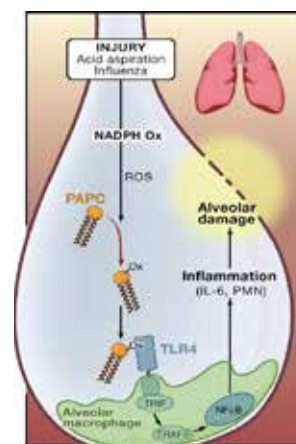


Fig. 1. Influenza A enters the lungs where it infects epithelial cells and then replicates and infects other cells (e.g. alveolar macrophages). Activation of these cells leads to the induction of the master transcription factors NFκB and results in the production of various cytokines (e.g. TNF-α) and chemokines that serve to perpetuate the inflammatory response through the recruitment of peripheral blood monocytes, macrophages, neutrophils and T cells into the airways. Activated macrophages and epithelial cells also release ROS, which cause lung inflammation, edema, tissue injury and lung dysfunction

Thomas R. Martin, and Mark M. Wurfel. Cell 133, April 18, 2008.

site of nervous system involvement, this damage can lead to such varied pathological processes as meningitis, neuritis, myelitis, encephalitis, vasculitis, or demyelinating disease (reviewed by Tyler and Nathanson, 2001; Love and Wiley, 2002).

The acute form of influenza was shown to result in the release of several biogenic amines (Han *et al.*, 2000; Jabody, 2002), production of interferon (Samuel, 1991; Cox and Hughes, 1999), activation of nitric oxide synthase and xanthine oxidase (Oda *et al.*, 1989; Akaike *et al.*, 1996; Peterhans, 1997a; Murphy *et al.*, 1998), stimulation of respiratory burst in phagocytic cells (Peterhans, 1997a, b) and accumulation of lipid peroxidation products in blood and target organs (Oda *et al.*, 1989; Schwarz, 1996; Peterhans, 1997a,b; Murphy *et al.*, 1998; Mileva *et al.*, 2000). Influenza infection activates resident phagocytes in the tissues to generate reactive oxygen species as part of the host's defense mechanism against virus replication. In order to protect the cells from virus-induced oxidative processes, host cells must have evolved some antioxidant mechanism that effectively scavenges the reactive oxygen species (Fig. 3).

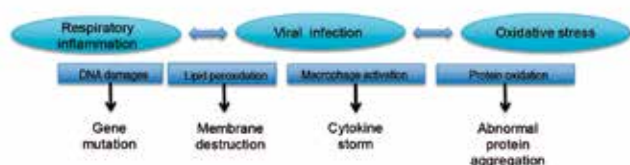


Fig. 2. Consequences caused by viral infections

The lipid-rich lung cell membranes are particularly susceptible to lipid peroxidation, an autocatalytic process that damages lipid-containing structures and yields reactive by-products as malondialdehyde (MDA) and 4-hydroxy-2-nonenal (HNE), which can covalently modify and damage cellular macromolecules. Their levels in the body are an adequate marker of oxidative damage (Fig. 4).

HNE and MDA are electrophilic species that can covalently modify and damage cellular macromolecules. At physiologic concentrations, HNE and MDA are potent regulators of cell growth and differentiation, affecting both cellular transcription and cell cycle progression (Ji *et al.*, 1998; Keller and Mattson, 1998; Poli and Schaur, 2000; Kakashita and Hattori, 2001). Oxidative cellular injury can cause cellular dysfunction and, when severe, this form of injury can cause cell death. Steady-state levels of oxidative tissue damage rep-

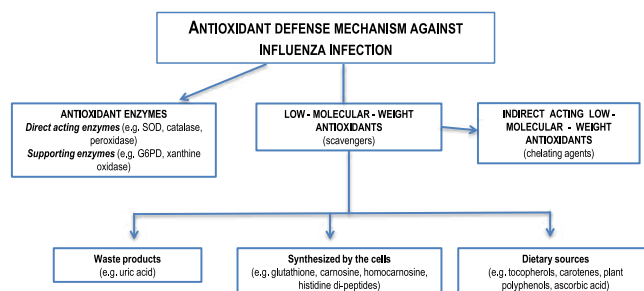


Fig. 3. Antioxidant defence mechanisms against influenza varus infection

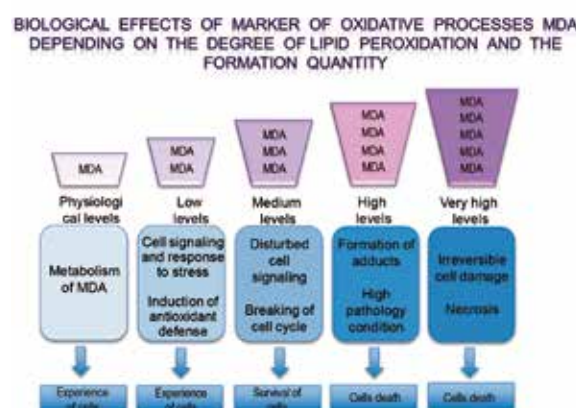


Fig. 4. Biological effects of biochemical marker of oxidative processes malondialdehyde (MDA) depending on the degree of lipid peroxidation and the formation quantity

resent a balance between rates of damage caused by pro-oxidant stimuli and rates of antioxidant and tissue repair mechanisms that decrease ROS/RNS levels and remove oxidatively damaged molecules (reviewed by Halliwell and Gutteridge, 1999; Halliwell and Whiteman, 2004).

The production of reactive oxygen species (ROS), particularly superoxide, is an important host defense mechanism for killing invading pathogens. However, excessive superoxide may be detrimental following influenza A virus infection. Influenza A virus infection causes a rapid influx of inflammatory cells, resulting in increased reactive oxygen species production, cytokine expression, and acute lung injury.

This is one of life's paradoxes: (i) molecular oxygen supports aerobic life: it plays an essential role for energetic metabolism and for respiration; (ii) oxygen radicals produced by one's own body are responsible for the cell defense system, destroying bacteria and viruses, decomposing chemical pollutants, and neutralizing toxins; (iii) almost equally they are involved in the ethiopathogenesis of numerous diseases and degenerative states due to oxygen-based reactive species called free radicals.

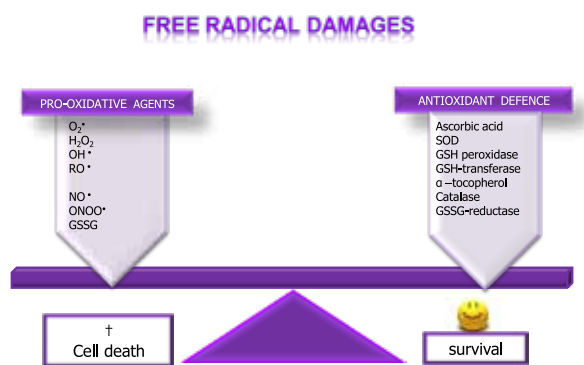


Fig. 5. Oxidative stress is defined as a state of:

- Abnormal generation of free radicals;
- Compensatory changes in the activities of antioxidant enzymes;
- Decreased levels of endogenous low molecular antioxidants (vitamin E, glutathione);
- Disturbed anti-oxidant/pro-oxidant balance;
- Disturbed redox state of organism.

Therefore, ROS are a necessary evil of aerobic life, being generated continuously during the process of respiration, but with the potential to cause oxidative deterioration of protein, lipid and DNA (Fig. 2). A range of different stress conditions elevates ROS generation. ROS damage is linked to serious degenerative conditions in humans. The cumulative production of ROS/RNS through either endogenous or exogenous ways is an important element of the condition called oxidative stress (Fig. 5). Oxidative stress corresponds to an imbalance between the rate of oxidant production and that of their degradation (Fig. 4). It is implicated as a pathogenic factor in a number of viral infections, including influenza viral infection (Oda *et al.*, 1989; Schwarz, 1996; Peterhans, 1997a,b; Murphy *et al.*, 1998; Mileva *et al.*, 2000). Oxidative stress caused by influenza virus increased vascular permeability and increased “fragility” of vessels leading to edema of the airway mucosa and lung tissue, multiple hemorrhages in the alveolar area, and interstitial lung as well as in virtually all the internal organs. The extent to which oxidative damage plays a beneficial role for the host by limiting viral replication is largely unknown. An enhanced understanding of the role of oxidative damage in influenza viral infection may lead to therapeutic strategies to reduce tissue damage during viral infection without impeding the host antiviral response (Valyi-Nagy *et al.*, 2005).

New therapeutic approaches for treatment of influenza

Early treatment of influenza might fail to prevent complications in some patients, particularly those infected with novel viruses such as the 2009

pandemic influenza A H1N1, avian influenza A H5N1 virus subtype, or the avian influenza A H7N9 virus subtype. Furthermore, treatment with one antiviral drug might promote the development of antiviral resistance, especially in immunocompromised hosts and critically ill patients. An obvious strategy to optimize antiviral therapy is to combine drugs with different modes of action. Because host immune responses to infection might also contribute to illness pathogenesis, improved outcomes might be gained from the combination of antiviral therapy with drugs that modulate the immune response in an infected individual.

Preventing lung injury induced by influenza with CR2 targeting complement inhibitor

The overreaction of the immune system is an important cause of patient mortality. Oda *et al.* pointed out in 1989 that symptoms of influenza are inflammatory injury as a result of immune activation by influenza virus, instead of being directly induced by influenza virus. The immune system is activated in case of invasion by influenza virus.

Complement is an important and conservative system of natural immunity, and provides pathways for rapid and effective elimination of invasive micro-organisms (Walport, 2001; Stoiber *et al.*, 2008). It is a “bridge” between natural immunity and acquired immunity. If the complement system is over activated, many complement components will be consumed, and the anti-infection ability of the body will be reduced; many active substances derived from the activation will induce a severe inflammatory reaction or tissue injury, resulting in a pathological process (Blach-Olszewska *et al.*, 2007). CR2 is the central molecule for the immune response regulation by the complement system. An effective CR2 targeting complement inhibitor can reduce mortality; significantly improve clinical symptoms (decreased weight, lung index and hemagglutination titer) and lung tissue inflammatory injury of a virus infected model group. Therefore in 2010, Zhang *et al.* hypothesized that a CR2 targeting complement inhibitor is expected to be an ideal drug for viral pneumonia.

Combination of neuraminidase inhibitor oseltamivir with immunomodulatory drugs

Sharma *et al.* (2013) evaluated the effect of the neuraminidase inhibitor oseltamivir in combination with immunomodulatory drugs *in vitro* and in a mouse model of influenza A H1N1 infection by determining survival rate, lung inflammation mark-

ers and histopathology. Sertraline and rolipram significantly improved survival in mice infected with a lethal dose of influenza A H1N1 virus. Prophylactic treatment resulted in survival rates of 40% (rolipram), 30% (oseltamivir), 0% (sertraline), 100% (rolipram/oseltamivir) and 70% (sertraline/oseltamivir). Treatment in a therapeutic setting (24 h post-infection) resulted in 80% (rolipram/oseltamivir) and 40% (sertraline/oseltamivir) survival. Sertraline and rolipram had no effect on virus replication *in vitro* and *in vivo*, but significantly reduced lung inflammation. A significant reduction in cellular infiltration (10-fold) along with inflammatory cytokines monocyte chemoattractant protein-1 (10-fold), interleukin-6 (5-fold) and regulated on activation normal T cell expressed and secreted (5-fold) was observed in the animals treated with the combination compared to oseltamivir alone. Lung histopathology of mice treated with combinations revealed significantly reduced consolidation, infiltration and alveolitis compared to oseltamivir alone. Rolipram and sertraline reduced H1N1 virus-induced lung inflammation and mortality. These data support further development of immunomodulatory agents for severe influenza.

Combination of neuraminidase inhibitor oseltamivir with low molecular antioxidants

The combined therapy of influenza is a question of particular interest. Garozzo *et al.* (2007) achieved up to 100% survival rate in mice infected with influenza virus and treated with combination of N-acetylcysteine (NAC) as a precursor of glutathione in a dose of 1000 mg/kg and oseltamivir in a dose of 1 mg/kg. The glutathione system is especially important for cellular defense against ROS. GSH reacts directly with radicals in non-enzymatic reactions and is the electron donor in the reduction of peroxides catalyzed by glutathione peroxidase (GPx) (Dringen *et al.*, 2000). Glutathione serves as the major scavenger of reactive oxygen species. Certain lymphocyte functions, such as the DNA synthetic response, are exquisitely sensitive to reactive oxygen species and, therefore, are favored by relatively high levels of glutathione. Even a moderate depletion of the intracellular glutathione pool has dramatic consequences for a variety of lymphocyte functions (Droge and Breitkreutz, 2000).

According to Dimitrova *et al.* (2016), in an experimental mice model of influenza type H3N2, the virus infection with 10 LD₅₀ causes 70% lethality. The monotherapy with oseltamivir in a dose of 2.5 mg/kg increases the survival rate from 30 to

70%. The monotherapy with S-adenosyl-L-methionine (SAM) in a dose of 50 mg/kg and 100 mg/kg does not increase the survival rate. It is a matter of interest that the combined therapy - SAM in both doses applied intraperitoneally combined with oseltamivir in a dose of 2.5 mg/kg daily in two intakes applied orally achieve 90% survival rate in mice infected with influenza virus. This work demonstrates the advantage of combining agents acting through different mechanisms – the antiviral drug oseltamivir and SAM as a precursor of the main antioxidant - glutathione.

The present study showed the positive effect of combining oseltamivir as a specific neuraminidase inhibitor of the influenza virus replication with S-adenosyl-L-methionine as a precursor of glutathione - the most abundant antioxidant in the body. The good results of the combination could be explained by the modulation of the host defense mechanisms and by the direct antioxidant effect of increased glutathione against oxidative stress associated with influenza infection.

These findings suggest that therapy with molecules converted into antioxidants in the body increases survival by modulating the host defense mechanisms and by a direct antioxidant effect against oxidative stress associated with viral infections. Those studies demonstrated the effectiveness of combining agents that act through different mechanisms - antiviral drug oseltamivir as specific neuraminidase inhibitor of influenza virus, and SAM and NAC as precursors of the most important antioxidant - glutathione.

To target the main processes involved in influenza pathogenesis, Galabov *et al.* (2016) studied the effects of oseltamivir and α -tocopherol combinations against influenza A/Aichi/2/68(H3N2) virus infection in mice. Oseltamivir was applied orally at three daily doses, 2.5, 1.25, and 0.625 mg/kg, in 5-day course post-infection. α -Tocopherol (120 mg/kg) was administered intraperitoneally. The results demonstrated pronounced dose-dependent synergistic antiviral effect of the combination α -tocopherol/0.625 mg/kg oseltamivir when α -tocopherol was administered simultaneously with oseltamivir: a 78% protection effect and lengthening of mean survival time by 3.2–4 days. Lung parameters showed a substantial decrease in infectious virus content ($\Delta\log_{10}$ =3.8/4.1) and a marked diminishment of lung index and pathology. The combination with 1.25 mg/kg oseltamivir manifested a marked protection, but less effect on lung parameters. The combination α -tocopherol/2.5 mg/kg

kg oseltamivir did not surpass the monotherapeutic effect of oseltamivir. When α -tocopherol was applied 5 or 2 days before infection, its combination with oseltamivir was ineffective.

Finally, oxidative stress and stress-mediated complications of influenza viral infection successfully respond to antioxidants or their precursors, including in monotherapy. However, it should be kept in mind that antioxidants are not antivirals, their function is more auxiliary, particularly in combination therapy with specific inhibitors of influenza infection.

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Phylogenetic Analysis of Ukrainian Isolates of RNA Viruses of Plants

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Abstract

The availability of analytical approaches allowing fast deciphering of nucleotide sequences enables phylogenetic analysis of viruses and establishing homology among their strains/isolates for unveiling the history of virus evolution.

This work was focused on phylogenetic analysis of the most important viruses infecting vegetable crops in Ukraine. 'Field' isolates of different plant RNA viruses were collected and analyzed from various agroecosystems. These included Potato virus Y (PVY), Cucumber mosaic virus (CMV), Watermelon mosaic virus 2 (WMV-2), Tomato mosaic virus (ToMV), Pepper mild mottle virus (PMMoV). Using MEGA6 software, phylogenetic trees were constructed, based on sequenced viral cDNAs corresponding to parts of the coat protein genes of the studied pathogens. Differing evolutionary trajectories were shown for some of the Ukrainian virus isolates.

The study of the molecular properties of viruses, and especially those of viral genomes, has fundamental importance (tracing phylogenetic relationships of a given virus with other members of the kingdom, its evolutionary history and trends), and is also of practical significance, as such data may further be used both for developing highly specific PCR-based diagnostic techniques as well as for forecasting possible alterations and novel properties of circulating viruses for prevention of disease spread and minimizing the aggressiveness of viruses.

Key words: ToMV, PMMoV, CMV, PVY, WMV2, coat protein gene, phylogenetic analysis

Резюме

Възможността за бързо разчитане на нуклеотидните последователности позволява филогенетичен анализ на вирусите и установяване на хомоложност между техните щамове и изолати с цел разкриване на вирусната еволюция. Дейността в представения материал е фокусирана към филогенетичния анализ на важни вируси, заразяващи овощни, зеленчукови и житни култури. Изследвани са изолати на вируси, съдържащи РНК, които са събрани в различни агроecosистеми. Изолатите са на причинителите на: зелената краставична мозайка (cucumber green mottle mosaic virus - CGMMV), сливовата шарка (plum pox virus - PPV), ипсилон виروزата по картофа (potato virus – Y, PVY), X-виروزата по картофа (potato virus X, PVX), ябълковата мозайка (apple mosaic virus - ApMV), ябълковите стъблени вдлъбнатини (apple stem pitting), ябълковото стъблено набраздяване (apple stem grooving virus), ябълковите хлоротични листни петна (apple chlorotic leaf spot virus - ACLSV), краставичната мозайка (cucumber mosaic virus - CMV), динената мозайка – 2 (watermelon mosaic virus – 2, WMV - 2), жълтата мозайката по тиквичките - зучини (zucchini yellow mosaic virus - ZYMV), доматиената мозайка (tomato mosaic virus - ToMV), пиперовото умерено прошарване (paper mild mottle virus - PepMMV) и пшениченото вджуджаване (wheat dwarf virus - WDV).

Чрез MEGA5 софтуер са съставени филогенетичните дървета на основата на секвенирани кДНК-и към части на кодиращите обвивния протеин гени на изучаваните патогени. Посочени са различни еволюционни траектории за някои украински вирусни изолати. Проучването на вирусите на молекулно ниво и по-специално на вирусните геноми (проследяването на родството на даден вирус с други представители на вирусното царство, неговата еволюционна история и насока на развитие) има и практическо значение. Например за диагностиката могат да бъдат развити високоспецифични PCR-базирани техники. Също така могат да бъдат предсказани изменения и нови свойства в циркулиращите вируси, за да бъде предотвратено разпространяването на болестите и намаляване на агресивността на вирусите.

Introduction

During recent years, plant virologists have witnessed an increased interest in the advance of knowledge at the level of population and similar ecology-oriented research. This trend is common for many traditional molecular biology studies. The development of a new generation of diagnostic methods for plant viruses (such as ELISA, RIA, RIPA, PCR, RT-PCR, etc.) during the last decade enabled a new level of studying the spread of plant viruses in the environment.

The relevance of this question lies in both its fundamental and practical significance. The determination of virus spread, mechanisms of virus transmission, natural range of host plants, and research on virus response to environmental changes makes it possible not only to more fully characterize a given representative of the Vira kingdom, but also to predict the emergence and development of viral diseases for developing sound strategies of combating viral infections. This includes search for resistant varieties, control of virus reservoirs and carriers, obtaining virus-free planting material, etc.

In Ukraine, major vegetable crops (such as tomato, cucumber, sweet pepper, pumpkin, squash, etc.) are mainly suffering from viral infections induced by Potato virus Y (PVY), Cucumber mosaic virus (CMV), Watermelon mosaic virus 2 (WMV-2), Tomato mosaic virus (ToMV), Pepper mild mottle virus (PMMoV), and these viruses were studied in the context of spread, relevance, biological and molecular properties, as well as phylogenetic relationships.

At present, ToMV is widespread in Ukraine and remains the point of interest due to the harm it causes to crops, particularly tomato and some other plants of the *Solanaceae* family (Silva *et al.*, 2011; Virus taxonomy, 2012). Until now, there has been no data on the phylogeny of ToMV isolates circulating in Ukraine.

The same is true for PVY and CMV infecting a wide range of cultivated and wild growing plants, both in open field and glasshouse conditions. Previously, CMV isolate of subgroup II was detected in infected pumpkin plants from Ukraine (Zitikaitė *et al.*, 2011). Subgroup attribution has been established based on the RT-PCR product size. However, sequences of these isolates have not been obtained and their comparison with other known strains and isolates has not been performed. In the current work, we also proceeded with strain attribution of Ukrainian isolates of CMV based on phylogenetic analysis of the partial sequences of the coat protein gene.

In Ukraine, WMV-2 has earlier been found

on plants (mostly marrow, pumpkin, zucchini, melon and cucumber) cultivated in open field conditions only. In addition to monoinfection, Watermelon mosaic virus 2 often circulates in mixed infections, typically induced by Cucumber mosaic virus (CMV), Zucchini yellow mosaic virus (ZYMV) and Cucumber aphid-born yellows virus (CABYV). Our previous results indicated that WMV-2 circulation in Ukraine showed some sort of cyclicity and irregularity. As Watermelon mosaic virus 2 is spread worldwide, it is of importance to analyze the homology of its Ukrainian isolates with reported WMV-2 isolates and strains, and to elucidate the possible ways of appearance of the Ukrainian isolates.

Pepper mild mottle virus (PMMoV) has only been discovered in Ukraine three years ago and was probably introduced with plant/seed material. Therefore, its phylogenetic properties have remained unknown but are highly relevant due to the epidemiological significance of the pathogen.

Considering all these issues, in the present study we focused on the phylogenetic analysis of the most important viruses infecting vegetable crops in Ukraine, which have still remained unexplored in evolutionary terms.

Materials and Methods

Plant samples were collected from different regions of Ukraine. The symptomatic samples were screened for the presence of viral antigens. Double-antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) was conducted using commercial test systems of Loewe (Germany). Plant material was homogenized in 0.1M phosphate buffered saline (PBS), pH 7.4, 1:2 (m/v). Plant components were removed by centrifugation at 5.000 g for 20 minutes at +4°C using centrifuge PC-6. The supernatant was used for further ELISA. DAS-ELISA was performed according to the manufacturer's recommendations. The results were checked at the wavelength of 405/630 nm using Termo Labsystems Opsis MR microplate reader (USA) with Dynex Revelation Quicklink software (Crowther, 1995).

Total RNA was extracted from naturally infected plant samples using RNeasy Plant Mini kit (Qiagen, UK). The results were confirmed by electrophoresis of nucleic acids in 1.5% agarose gel.

For CMV, a two-step reverse transcription reaction (RT-PCR) was accomplished using two specific primers complementary to the coat protein gene and producing the amplicon with expected

size of 500 bp (Bariana *et al.*, 1994):

forward primer –

5' TATGATAAGAAGCTTGTTCGCGCA-3';

reverse primer –

5' TTTTAGCCGTAAGCTGGATGGACAACCC-3'.

For ToMV, specific primers covering the coat protein (CP) gene were used (product size – 700bp) (Letschert *et al.*, 2002):

forward primer –

5' CGGAAGGCCTAAACCAAAAAG-3';

Tob-Uni1 primer –

5' ATTTAAGTGGAGGGAAAAACACT-3'.

For PMMoV, we used primers specific to a part of the coat protein gene (product size of 387 bp) (Jarret *et al.*, 2008):

forward primer –

5' TAC TTC GGC GTT AGG CAA TC-3',

reverse primer –

5' GGA GTT GTA GCC CAG GTG AG-3'.

PVY-specific primers covering the coat protein (CP) gene were used (product size – 569 bp) (Budzanivska *et al.*, 2014):

(As) 5'- CAAACCATAAGCCCATTCATC-3'

(S) 5'- GCACCA AATCAG GAG ATT CTA CT-3'.

For WMV2, primers specific to the CP gene of genomic RNA of French strain of WMV2 (Zohren, 2011) were used (product size – 825bp):

forward primer –

5' GAATCAGTGTCTCTGCAATCAGG-3';

reverse primer –

5' ATTCACGTCCCTTGCAGTGTG-3'.

Purified amplicons were sequenced using Applied Biosystems 3730x1 DNA Analyzer with Big Dye terminators, version 3.1 (Applied Biosystems, USA).

The aligned sequences of the parts of the coat protein gene of the respective virus isolates were compared with published sequences of virus strains available in the GenBank database using NCBI/BLAST (<http://www.ncbi.nlm.nih.gov/>). The phylogenetic analysis was conducted using MEGA 5 (Tamura *et al.*, 2011). The phylogenetic trees were constructed using the Neighborhood Joining and Maximum Likelihood methods (Kimura, 1980; Felsenstein, 1982).

Results and Discussions

ToMV

Comparison of 480bp-length CP sequences using NCBI/BLAST (<http://www.ncbi.nlm.nih.gov/>) showed that the obtained isolate (ToMV-ukr3) shares the highest identity with the group of tobamoviruses mainly infecting solanaceous plants (To-

mato mosaic virus, Tobacco mosaic virus (TMV), Tomato mottle mosaic virus (ToMMV), Pepper mild mottle virus (PMMoV)). The criteria for *Tobamovirus* species differentiation determine that less than 90% nucleotide sequence identity is considered a new species (Virus taxonomy, 2012). The Ukrainian isolate showed minor differences and revealed 96-99% nucleotide identity with different ToMV strains, whereas ToMV-ukr3 shared much less identity with TMV (74-79%), PMMoV (71-73%), and ToMMV (85%). These results confirmed the attribution of the obtained isolate to ToMV.

A phylogenetic tree was built using Maximum Likelihood method Mega 6 software (Fig. 1).

On the ML tree, the Ukrainian isolate and all strains under study excepting strain ToMV-A356 were clustered together. They shared a common ancestor and close phylogenetic relationships. High homology percentage values were determined between these sequences: 98-99%. The Ukrainian isolate grouped with strains ToMV-1-2, ToMV-G26 and ToMV-G6, homology values with them were approximately 99% at nucleotide level. Strain ToMV-A356 represents the lowest homology percentage (96%) with ToMV-ukr3. Tobamoviruses demonstrate strong correlation with their angiosperm hosts (Stobbe *et al.*, 2012) supporting the suggestion that this low rate of homology could be explained by the fact that strain ToMV-A356 was extracted from *Centaurea sp.*, member of the *Asteraceae* family.

PMMV

Until today, 13 strains of Pepper mild mottle virus, a novel and emerging pathogen in Ukraine, have been found to infect different hosts in various countries (Li *et al.*, 2013). Here, we phylogenetically analyzed two isolates of PMMoV found in sweet pepper and tomato based on their CP gene sequence. We obtained cDNA of expected size of 387 bp, which was further used for sequencing and construction of a phylogenetic tree (Fig. 2). The phylogenetic tree was constructed using the Neighbor-Joining method.

Obviously, the Ukrainian isolates of PMMoV collected from sweet pepper and tomato belong to the cluster composed of viruses found in Spain and Japan. Both Ukrainian isolates of PMMoV were highly homologous to each other (>99%) and thus belonged to the same strain.

CMV

A phylogenetic tree was constructed using aligned nucleotide sequences of the CP gene of various CMV strains isolated from different coun-

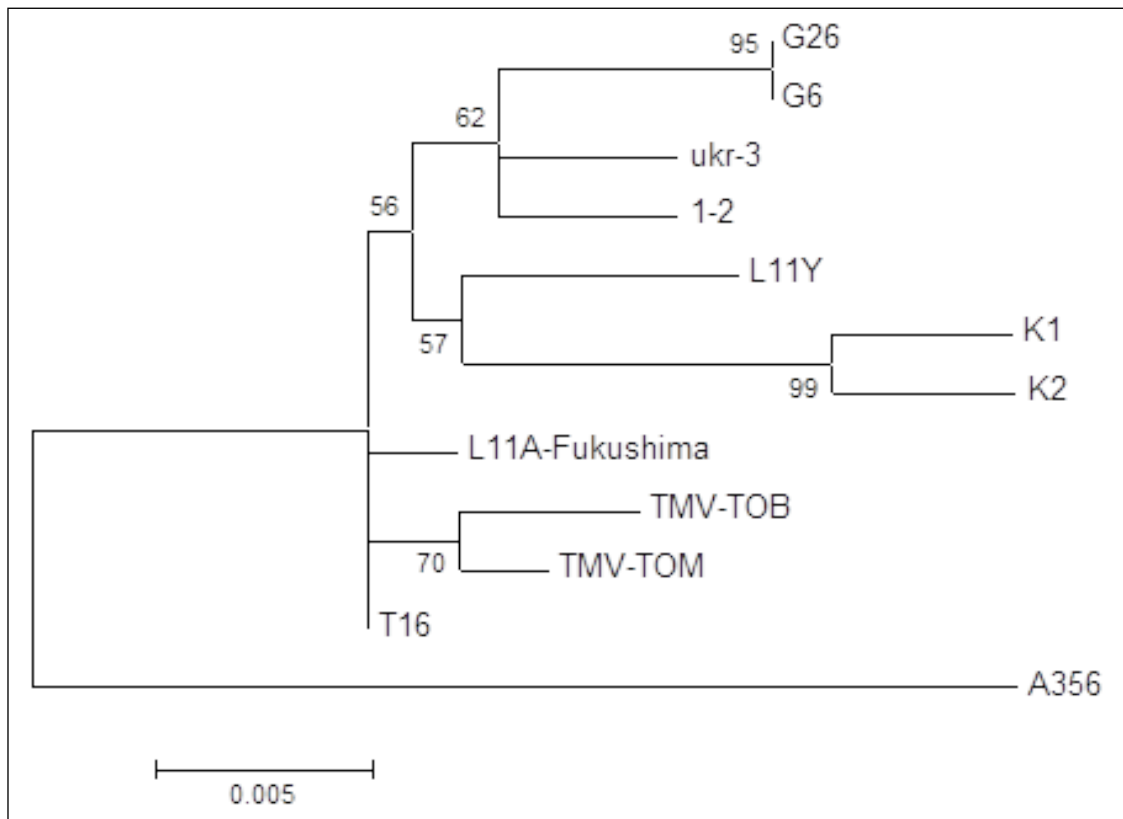


Fig. 1. Phylogenetic tree based on entire coat protein gene sequence of selected ToMV strains and Ukrainian isolate

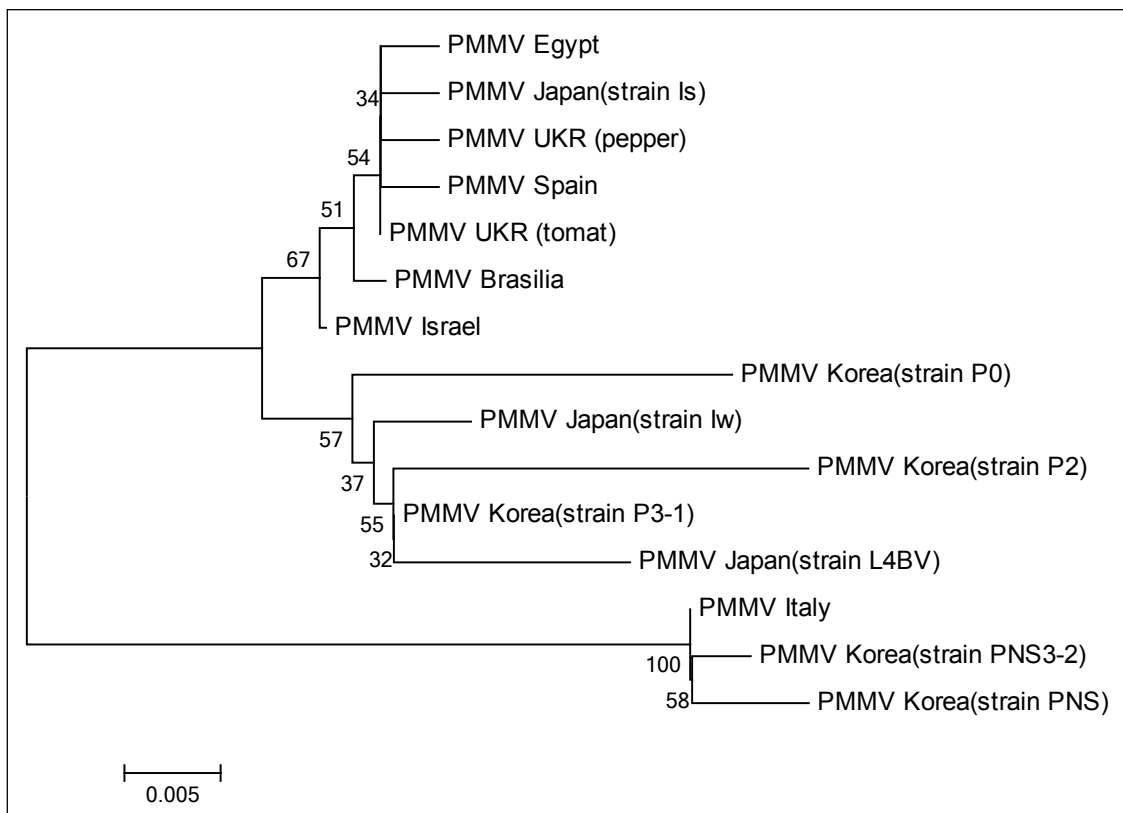


Fig. 2. Phylogenetic tree (NJ) showing evolutionary relations of Ukrainian isolate of PMMoV with published virus sequences (based on the coat protein gene)

tries and Ukrainian isolates. Phylogenetic analysis indicated three distinct clusters corresponding to subgroups IA, IB and II (Fig. 3).

CMV strains belonging to subgroup II formed a different cluster on the phylogenetic tree, well separated from the members of subgroup I. Within subgroup I, Ukrainian CMV isolates showed close phylogenetic relationships rather with the members of subgroup IB than IA, which formed a separate cluster.

Ukrainian isolates shared 79-99% nucleotide homology with the strains reported from all over the world (Table 1). According to literature data,

members of the same group share more than 90% homology. The homology between strains from I and II subgroups was approximately 69-77% (Palukaitis and García-Arenal, 2003). The nucleotide sequences shared 92-94% similarity among IA and IB subgroup strains (Kumari *et al.*, 2013). The Ukrainian isolates were phylogenetically most related to each other and to the members of subgroup IB (>90% nucleotide homology). However, they were distinct from the subgroup IA and subgroup II strains. The members of subgroup II showed the lowest nucleotide homology (79-82%) with the studied Ukrainian isolates of CMV.

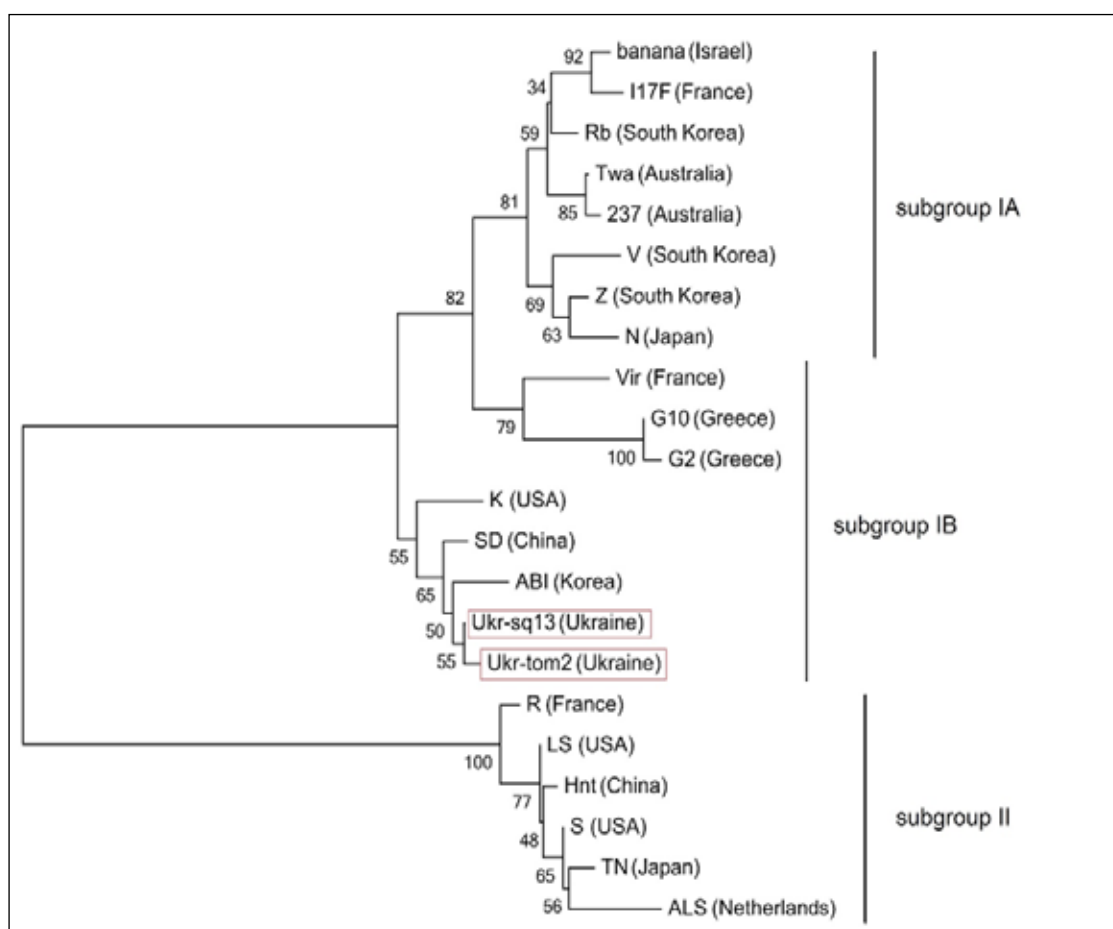


Fig. 3. Phylogenetic tree based on partial coat protein gene sequence of selected strains and Ukrainian isolates of CMV. Bootstrap values are shown above branches

The phylogenetic analysis of partial sequences of the CP gene of Ukrainian isolates of CMV revealed their highest homology and close relationships with strains ABI and SD from Korea and China. They shared approximately 98-99% homology. The infrequent amino acid substitutions revealed the high similarity in this gene region. The protein sequence of ABI strain is different from those of Ukr-sq13, Ukr-tom2, and SD, and has two amino

acid substitutions (YA – IT corresponding to 601, 602 positions). Sequence comparison of Ukrainian isolates showed their high similarity. Nucleotide analysis confirmed two synonymous nucleotide substitutions in positions 1612 (T (Ukr-sq13) – C (Ukr-tom2)) and 1946 (G (Ukr-sq13) – A (Ukr-tom2)). However, we were unable to identify any amino acid substitutions when comparing two Ukrainian isolates of CMV. Based on the obtained

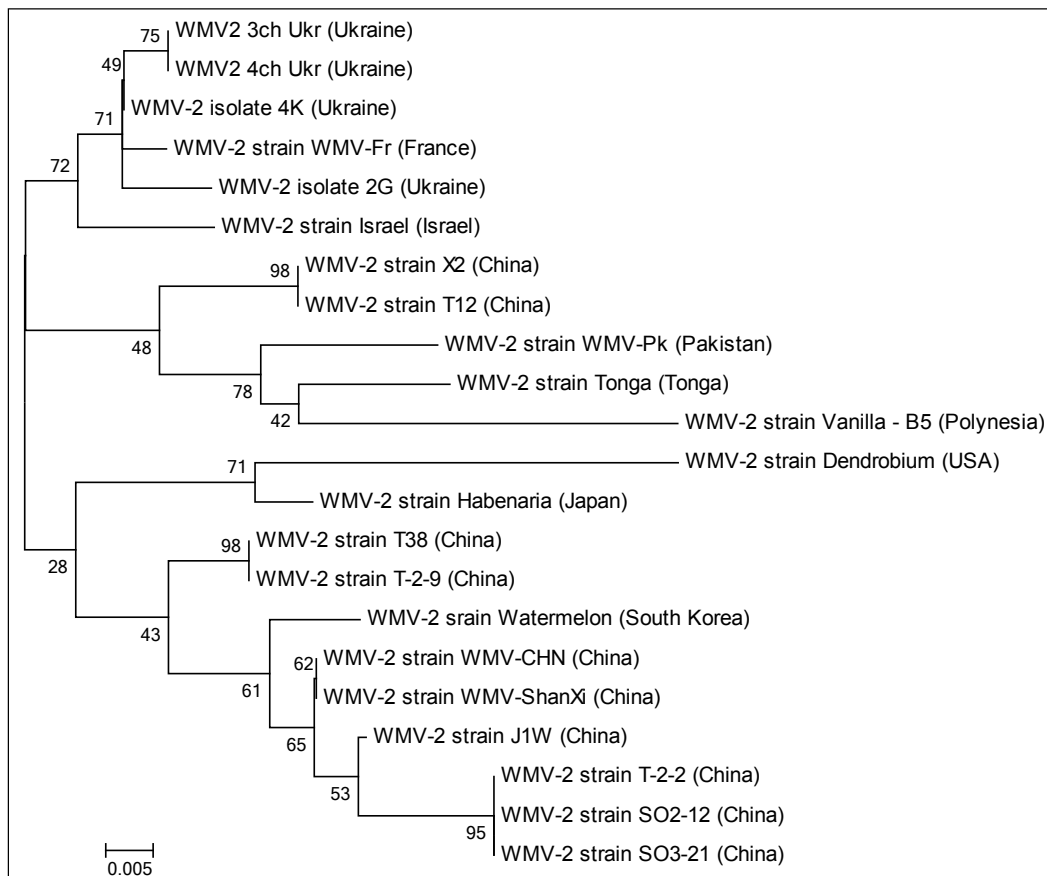


Fig. 4. Phylogenetic tree based on coat protein gene sequence of selected strains and Ukrainian isolates of WMV-2 (Maximum Likelihood, Jukes-Cantor model, 1000 bootstrap replications). Bootstrap values are shown above branches

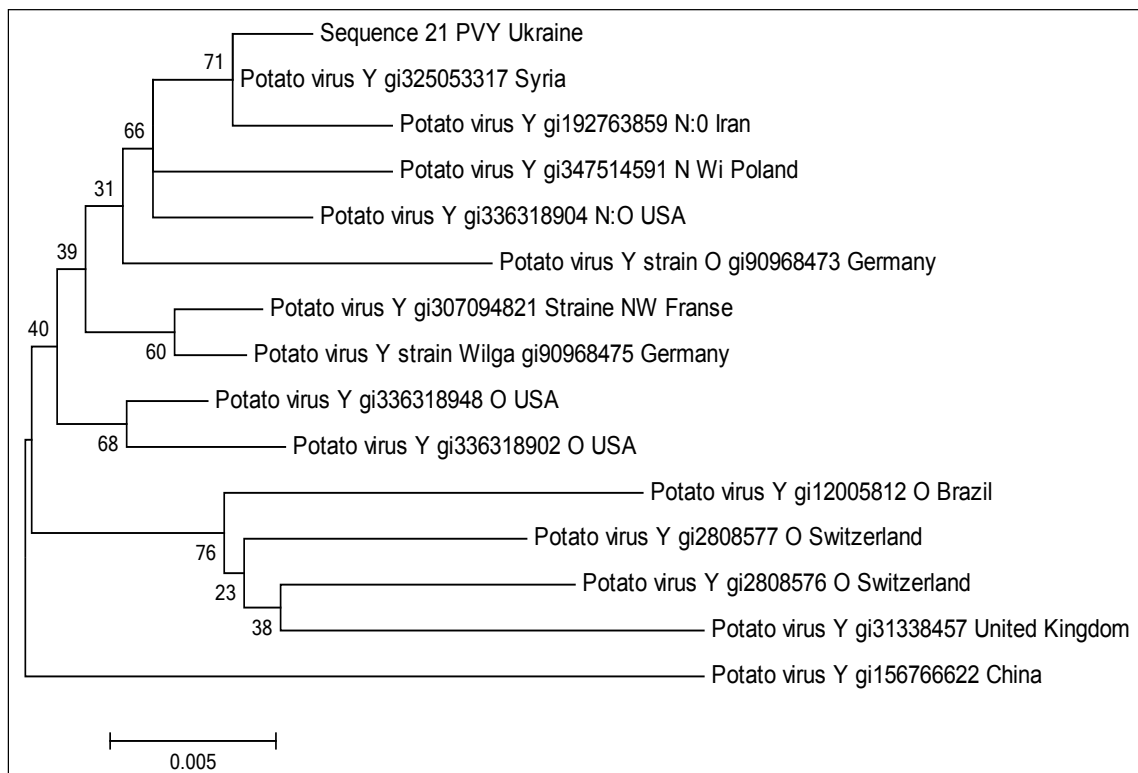


Fig. 5. Evolutionary relationships of the Ukrainian PVY isolates with PVY isolates/strains deposited in the GenBank (NCBI, USA). Evolutionary history has been reconstructed, using Neighbour Joining method (Saitou and Nei, 1987) for nucleotide sequence of the coat protein gene. Evolutionary distances were calculated using p-distance method (Tamura *et al.*, 2011) for 1000 bootstrap replications (number of base changes per site)

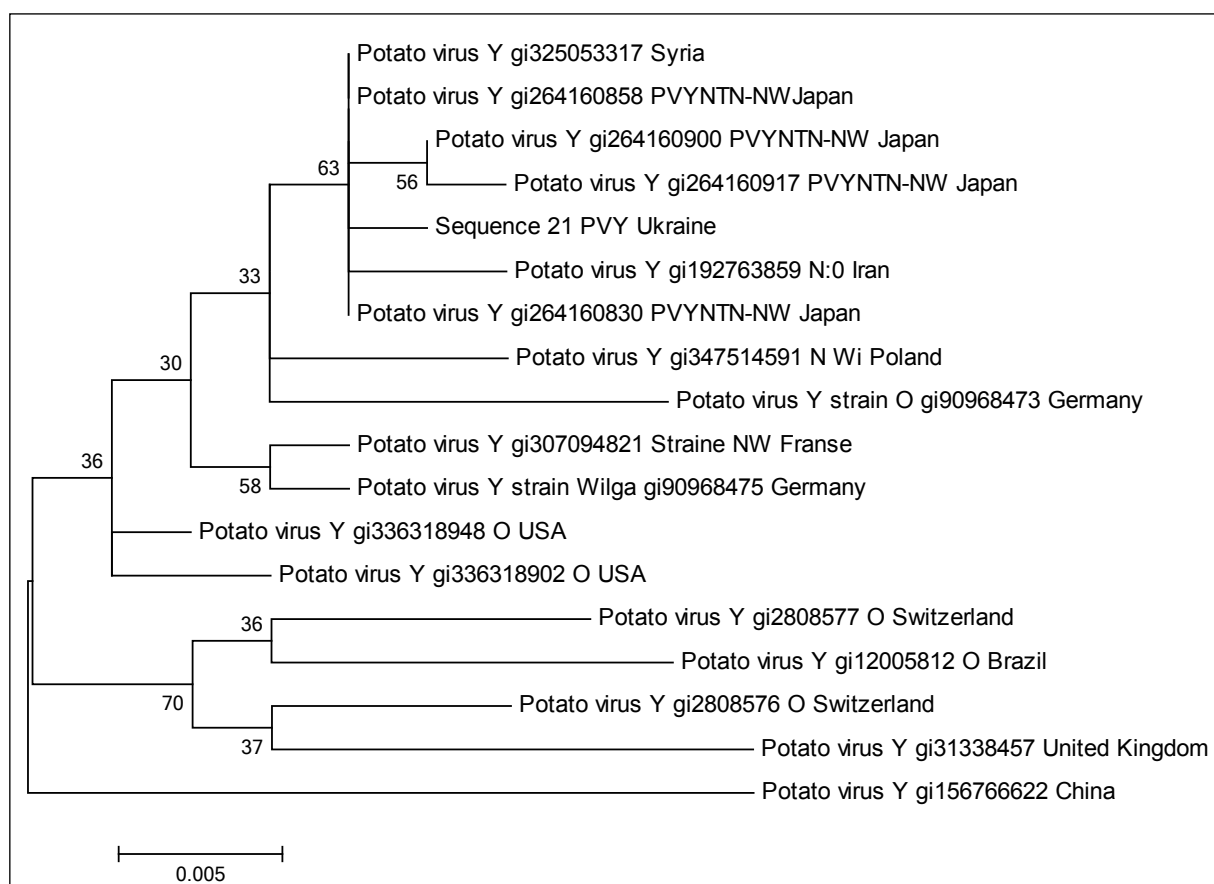


Fig. 6. Molecular phylogenetic analysis of nucleotide sequences of the PVY coat protein gene using Maximum Likelihood method (Felsenstein, 1982).

results, these Ukrainian isolates of CMV were identified as belonging to the subgroup IB. Sequence data have been submitted to NCBI, accession numbers KJ921838 and KJ921837 for Ukr-tom2 and Ukr-sq13 isolates, respectively.

WMV2

After sequencing the cDNAs of Ukrainian WMV-2 isolates, the achieved nucleotide sequences (designated WMV 2-2g-Ukr and WMV 2-2k-Ukr) were compared both among themselves and with other known strains and isolates of WMV-2.

A phylogenetic tree was built using Maximum Likelihood method Mega 6 software and Jukes-Cantor model (Fig. 4). The sequences were grouped into three separate clusters.

Evidently, Ukrainian isolates WMV2-3ch-Ukr, WMV2-4ch-Ukr, 2G, and 4K were grouped in a single cluster with a homology of 97.8-98.9%, confirming low level of variability of Ukrainian isolates of Watermelon mosaic virus 2. The Israeli strain 'Israel' and the French strain WMV-Fr belonged to the same cluster and showed a high level of homology (96.6-97.8%) with the Ukrainian isolates.

The other two clusters contained WMV strains from Pakistan and the Pacific Ocean region

(87.6-92.1% homology to Ukrainian isolates), and from USA, Japan, China and South Korea (89,9-93,3% homology with the Ukrainian isolates).

Therefore, the Ukrainian population of the virus is highly homologous and Ukrainian isolates are related to strains from France and Israel, which may well reflect their origin. Thus, WMV-2 may have been brought to Ukraine from the Mediterranean or Middle East regions.

PVY

RT-PCR yielded cDNA corresponding to the part of the CP gene of Ukrainian isolate of PVY. This cDNA was further sequenced and compared with known sequences of PVY isolates and strains published in the GenBank (with some of them more than 600 of such sequences are available).

These relationships are even more obvious when analyzed using phylogenetic trees constructed for nucleotide sequences of the CP employing NJ method (Fig. 5).

Interestingly, both nucleotide sequence- and amino acid sequence-based phylogenetic trees for PVY CP demonstrate highly similar (though not 100% identical) position of the Ukrainian isolate in relation to others. In both cases we can see a separate cluster, including recombinant isolates (strains)

from Syria and Iran; at the same time, PVY USA N:O and Poland N-Wi strains form another closely related small cluster.

To make a conclusion on the evolutionary history of these isolates, an ML approach was used on the basis of Tamura-Nei model. A tree with high logarithm of likelihood was constructed for 1000 bootstrap replications. Branch length corresponds to the number of changes per site.

As shown on Fig. 6, the phylogenetic tree constructed using ML method underlines that the main tendencies remain the same. It can be concluded that the Ukrainian PVY isolates and PVY isolates from Syria and Iran descended from a single ancestor.

The difficulties with interpretation of the obtained results for Ukrainian PVY isolates had not been anticipated and thus were rather unexpected. A state-of-the-art study (2006-2013) and previous (1992-2005) literature sources confirm the absence of unanimous opinion among authors regarding the unified nomenclature for strains and isolates of PVY (in particular, those found in potato). The comparison of phylogenetic trees published by different researchers is not an easy task as (most often) different 'source' virus sequences (both nucleotide and amino acid) have been used for analysis. Apparently, the recombination among the PVY genomes may lead to the development of novel variants of the virus, which may have differing phenotype (without any significant correlation with the coding sequences). In turn, phenotypic appearances will depend on the specific species and cultivar of the virus-infected plant.

As far as our work has not been aimed at studying phenotypic variations of Ukrainian isolates of PVY, our conclusions are based exclusively on the comparative analysis of nucleotide sequences in the virus genome (the coat protein gene of PVY). Phylogenetic relationships based on the CP gene of PVY and explored using the ML method may be used as an indirect index of relationships for full-size genomes of potyviruses (Roossinck, 1997) and for establishing species of viruses for which only CP gene sequences are available.

Our conclusions regarding close relationships between the Ukrainian PVY isolate and isolates from Syria and Iran, and about their putative common origin is strongly supported by the fact that these isolates are closely positioned in the other phylogenetic trees (PVY_Siria, PVY_Iran, PVY_China 2011). Following this lead and according to Chikh-Ali *et al.* (2007), it is viable to suggest that

the Ukrainian isolate of PVY belongs to the strain group O, subclade N:O (using another isolate classification proposed by Saitou and Nei, 1987) – to the subclade O N-Wilga N:O.

Conclusion

From the current work it can be inferred that the Ukrainian isolate of ToMV is not an emergent strain able to break the resistance in plants (Yamamoto *et al.*, 2002). Thus, known strategies for control of tomato diseases caused by ToMV may be applied. Phylogenetic analysis of cDNA corresponding to the CP gene of PMMoV indicates that it belongs to clusters composed of viruses found in Spain and Japan.

Despite the supposed high rate of CMV evolution and adaptation (Roossinck, 2002), phylogenetic analysis of two Ukrainian isolates of CMV reveals that their homology exceeds 95%, i.e. that these isolates belong to the same strain. In our opinion, it is of special interest as these isolates have been found in totally different host plants, tomato and squash, which belong to distant families.

Another important phylogenetic finding is that Ukrainian isolates of CMV are attributed to the subgroup IB of CMV strains. The Ukrainian isolates are mostly related to strains ABI and SD from Korea and China, respectively. Initially, the members of subgroup IB were found in (and thought to be restricted to) East Asia. Later on, subgroup IB strains of CMV were shown as widespread in Iran (Arafati, *et al.*, 2013), although subgroup IA isolates were also detected (Nematollahi *et al.*, 2012). The occurrence of subgroup IB isolates of CMV suggests that they may have been introduced in Ukraine either by seed material or through exported fresh food products. In addition, we cannot reject the possibility of virus 'naturally migrating' from Iran (or other neighboring regions) into Ukraine as transported by aphids or birds.

Strangely, we have not found subgroup IA isolates of CMV in Ukraine. These are more virulent strains and isolates, which are considered to be common worldwide, including Europe. According to previous research data (Zitikaitė *et al.*, 2011) and our findings, the CMV population consists of isolates of II and IB subgroups in Ukraine. Having registered severe symptoms on collected plants, we expected that they were induced by a virulent 'form' of the virus. Surprisingly, both isolates fall into subgroup IB strains of CMV. In the view of aforesaid, we deem that the obtained results rather reflect the lack of CMV monitoring in Ukraine than

reveal atypical virus spread in the region. The data also indicate that the severity of the virus-specific symptoms in field conditions may not be directly related to the degree of virulence of a given virus isolate, requiring more research of CMV biology, epidemiology and evolution.

Phylogenetic analysis of WMV-2 based on its CP gene indicates that the Ukrainian population of the virus is highly homologous and that Ukrainian isolates are related to strains from France and Israel, which may well reflect their origin. Thus, WMV-2 may have been brought to Ukraine from Mediterranean or Middle East regions (or vice versa).

Phylogenetic analysis of nucleotide sequence of the CP gene of the Ukrainian PVY isolate demonstrates that it is a recombinant virus capable of phenotypic appearances typical of both ordinary (O) strain and necrotic (N) strain, depending on the specific conditions of a plant virus infection development. The Ukrainian isolate of PVY has a significant mutational (recombinational) potential and in the future may therefore change its biological properties (in terms of the host range, for instance). This information may be of use for potato breeders as employment of wild potato varieties and its relatives in selection programs may induce unexpected 'response' of different PVY strains in new cultivars.

The established trend of low genetic variability of viruses, despite the high mutational ability of RNA viruses, indicates the impact of negative selection to maintain the stability of viral nucleotide sequences. Analysis of the phylogenetic trees topology shows the direction of isolation and low probability for the formation of a Ukrainian strain with new properties. Our data have shown high genetic conservatism for most populations of plant viruses that favors the modern hypothesis of evolution for plant RNA viruses. A high relationship level between all Ukrainian isolates has been revealed, indicating their high homogeneity.

Acknowledgements

The authors are grateful to Dr. I.I. Boubriak for his invaluable support and technical assistance.

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Studies on Malignant Catarrhal Fever in Wild Ruminants

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Abstract

Wild and domestic animals inhabiting Sofia and Varna zoos showing clinical symptoms of malignant catarrhal fever (MCF) were investigated. Clinical and pathological studies were carried out with 16 wild and 8 domestic ruminants. Typical clinical symptoms for MCF were confirmed. Samples for virus isolation and molecular biological investigations were obtained from dead animals. Ten percent suspensions from the lymph nodes, spleens, lungs, brain samples, and buffy coats in phosphate buffered saline (PBS) were used for viral isolation. Primary and permanent cell cultures: rabbit kidney, Madin Darby bovine kidney (MDBK), embryonic bovine trachea (EBTR). Bovine trachea (TTr) and VERO cells were used. Viruses with peculiarities of herpesviruses were isolated from bison, yak, camel, Cameroon goats and hippopotamus on MDBK cell culture. The isolated strains were confirmed as MCF viruses by classical and nested polymerase chain reaction (PCR). The size of the amplicons after classical PCR was 422 bp, and 238 bp after nested PCR.

Thirty seven serum samples originated from 17 sheep, 13 Cameroon goats, 5 mouflons and two bison were investigated serologically by a competitive-inhibition ELISA for spread of MCF. In 21.4% of sheep sera, 30.7% of Cameroon goat sera, and 50% of bison sera antibodies against MCF were found. The total distribution of MCF antibodies in wild and domestic ruminants was 27%.

Key words: Malignant catarrhal fever, wild ruminants, PCR, serological investigation, competitive inhibition ELISA

Резюме

Изследвани са диви и домашни животни обитаващи зоопарковете в София и Варна и показали клинични признаци на злокачествена катарална треска (ЗКТ). Клинични и патологоанатомични изследвания са извършени на 16 диви и 8 домашни преживни животни. Потвърдени са типичните за ЗКТ клинични симптоми. От умрелите животни са получени проби за вирусна изолация и молекулярно биологични изследвания. За вирусна изолация са използвани 10% суспензии от лимфни възли, слезки, бели дробове, мозъчни проби и съсирена кръв във фосфатно буферен разтвор (PBS). За вирусна изолация са използвани първични и постоянни клетъчни култури (КК) - заешки бъбрек (ЗБ), Мадин Дарби говежди бъбрек (МДБК), ембрионална бовинна трахея (ЕБТР) и бъбреци от зелена африканска маймуна (VERO) клетки. Вируси с характеристики на херпесвируси са изолирани от бизон, гаур, як, камила, камерунска коза, хипопотам върху КК МДБК. Изолираните щамове са потвърдени като вируси на ЗКТ чрез класическа и нестед полимеразно верижна реакция (PCR). Ампликоните след класическата PCR бяха с големина 422 bp, а след нестед PCR с големина 238 bp. За установяване на разпространението на ЗКТ чрез компетитивна инхибираща ELISA са изследвани серологично 37 серумни проби от 17 овце, 13 камерунски кози, 5 муфлони и два бизона. В 21,4% от овчите серуми, 30,7% от камерунските кози и 50% от бизоните бяха установени антитела срещу вируса на ЗКТ. Общото разпространение на антителата срещу вируса на ЗКТ при изследваните животни беше 27%.

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Introduction

Malignant catarrhal fever (MCF) is a fatal virus disease in wild and domestic animals. The disease has been observed in different captive ruminants in zoological collections. In some species, such as bison and some deer, MCF is acute and highly lethal, capable of affecting large numbers of animals. In farm-reared white tiled deer and American bison, the disease is a major threat and causes high economical losses. The etiological agent of MCF belongs to subfamily *Gammaherpesvirinae*, genus *Macavirus* from *Herpesviridae* family. Five gamma herpesviruses associated with MCF are ascribed to the genus *Rhadinovirus* - *Alcelaphine herpesvirus*-1 (AIHV-1) (Plowright *et al.*, 1960; Roizman, 1992), *Alcelaphine herpesvirus*-2 (AIHV-2) (Klieforth *et al.*, 2002), *Ovine herpesvirus*-2 (OvHV-2) (Li *et al.*, 1995; 1996; 1999; Muller Doblies *et al.*, 1998; Collins *et al.*, 2000), *Caprine herpesvirus*-2 (CpHV-2) (Crawford *et al.*, 2002; Keel *et al.*, 2003; Li *et al.*, 2001, 2003) and one virus, yet unnamed, causing MCF in white-tailed deer (MCFV-WTD) (Li *et al.*, 2000).

Two forms of MCF are known: African or wildebeest associated MCF (WD-MCF), and European or sheep associated MCF (SA-MCF). The carriers of MCF viruses in Africa are wildebeest (AIHV-1), in Europe are sheep (OvHV-2), and goats (CpHV-2). With accumulation of data on genomic peculiarities of different MCF agents, it has been determined that the viruses causing both forms of MCF are closely related, however, they are different viruses (Bridgen *et al.*, 1989; Reid, 1991; Ensler *et al.*, 1997; Coulter *et al.*, 2001). In ruminants inhabiting zoos, the MCF virus spreads from the wildebeest or sheep to susceptible animals. Wildebeest calves are infected by intrauterine transmission from the female or shortly after birth by horizontal transmission of the virus and spread the MCF infection for a period of 3-4 months. The way of infection of new born lambs is not fully clarified. Lambs are infected within 3-6 months of birth by aerosol transmission from older sheep and shed the virus until 9 months of age. The epizootology of caprine herpesvirus 2 is similar to OvHV-2. In European breeds of cattle (*Bos taurus*), the MCF disease is seen sporadically and is highly fatal. In other species, bison, Bali cattle, and cervid species (eg, white-tailed and Pere David's deer), the disease is highly contagious. Bison and deer are more susceptible to the disease than cattle, and the virus can remain in a latent form. Therefore, recrudescence of the infection is possible. The MCF infection is

lifelong in surviving animals.

In the autumn of 2014, clinical signs typical of MCF in wild big and small ruminants with high mortality rate were observed in Sofia and Varna zoos. The aim of the study is to identify the cause of the high mortality in wild animals and to characterize the agents responsible for the mortality.

Material and methods

An epidemiological investigation was performed in two zoos - Sofia and Varna. The time of outbreaks of disease, the number and type of affected wild animals, the duration of observed clinical symptoms and the time of deaths after onset of disease, the import of new animals in the zoo, contacts between wild and domestic animals, the type of rearing, type of foods and water supply and performed veterinary measures as treatment against bacterial and parasitic disease, disinfection, etc. were investigated.

A clinical investigation was performed with 15 wild and 8 domestic animals - 2 yaks, 4 bison, one camel, 3 gaurs, 2 capricorns, 2 Cameroon goats and one hypopotamus. During the anamnestic studies, changes in the animals' general state, clinical symptoms typical of the disease, temperature, changes in the eyes, central nervous system, respiratory tract, superficial and mesenteric lymph nodes, skin and mucous membrane were examined. The animals with clinical symptoms typical of MCF were pathologically studied after death - one yak, four bison, one gaur, two Cameroon goats and one hypopotamus. The materials for histological investigation were conserved in 10% buffered formalin and afterwards processed according to the methods of Lillie (1965).

Virological samples from the spleen, lymph nodes, lungs, liver, kidney, brain, and buffy coats from diseased wild and domestic ruminants were investigated. The samples were kept at 4°C till delivery in the laboratory and were preserved with glycerin. The samples were squeezed and a 10% organ suspension was prepared in phosphate buffered saline (PBS) pH 7.2. Penicillin 200 UI, Streptomycin 200 µg/mL or Gentamycin 20 µg/mL antibiotics were added to the samples. Afterwards the samples were centrifuged at 4000 rpm/15 min. Clear supernatant was used for inoculation of primary and permanent cell cultures. Primary rabbit kidney, primary lamb testicle and permanent cell culture Madin Darby bovine kidney (MDBK), embryonic bovine trachea (EBTR), bovine trachea (BTr) and green monkey kidney (VERO) were used for iso-

lation and identification of MCF viruses. Minimal Essential Medium (MEM), with Hank's, or Earle salts, Penicillin 100 IU/mL, Streptomycin 100γ/mL antibiotics, L glutamine, 0.2 M/L, sodium bicarbonate 7.5% for pH 7.4 of media and fetal calf serum (FCS) 10% were added as growth mediums. The maintenance medium was the same as the growth medium, but with a lower amount of FCS – 2%. The tubes with cell cultures were inoculated in three ways: on cell suspension, on a 24-hour-monolayer, or onto a fresh 3-4-hour-monolayer with dimethyl sulfoxide at a concentration from 0.5 to 1%. The tubes were placed for roller cultivation.

Titration of isolated viruses were performed by the methods of Reed and Muench (1938). The biochemical investigation of isolates - type of nucleic acids, presence of lipid envelope and sensitivity to low and high pH was carried out using the methods of Payment and Trudel, (1993). *Paramyxovirus parainfluenzae* 3, strain *Svetovrachene*, was used as a heterologous strain, which was treated in the same manner as the virus isolates from wild ruminants.

The isolated viruses were confirmed as MCF viruses after classical and nested polymerase chain reaction (PCR) by the methods described by Li *et al.* (1995). For determination of the MCF virus type (AIHV 1 or OHV 2), PCR with primers multiplying AIHV 1 was also used (Hsu *et al.*, 1990).

Thirty seven serum samples from wild and domestic zoo-inhabiting animals were investigated serologically. The investigation was performed by a microvirus neutralization test as described by Dilovski *et al.* (1982) against malignant catarrhal fever virus isolated from gaur with virus titer $10^{6.7}$ TCID₅₀/mL. The same samples were investigated by a competitive-inhibition ELISA by the methods described by Li *et al.* (1996).

Results

The epizootological investigation determined that 12 big and small ruminants were affected. In the same year, antelopes had been imported from a Romanian zoo, and a camel from a private owner. After six months one bison was found dead, and as the disease continued another 11 animals died within a period of two months. The bison and other big ruminants inhabited a place which was in close proximity with the premises inhabited by the antelopes and domestic sheep. The big and small ruminants were looked after by the same staff fed with the same food. Special immune-preventive actions, apart from deworming, were not carried out.

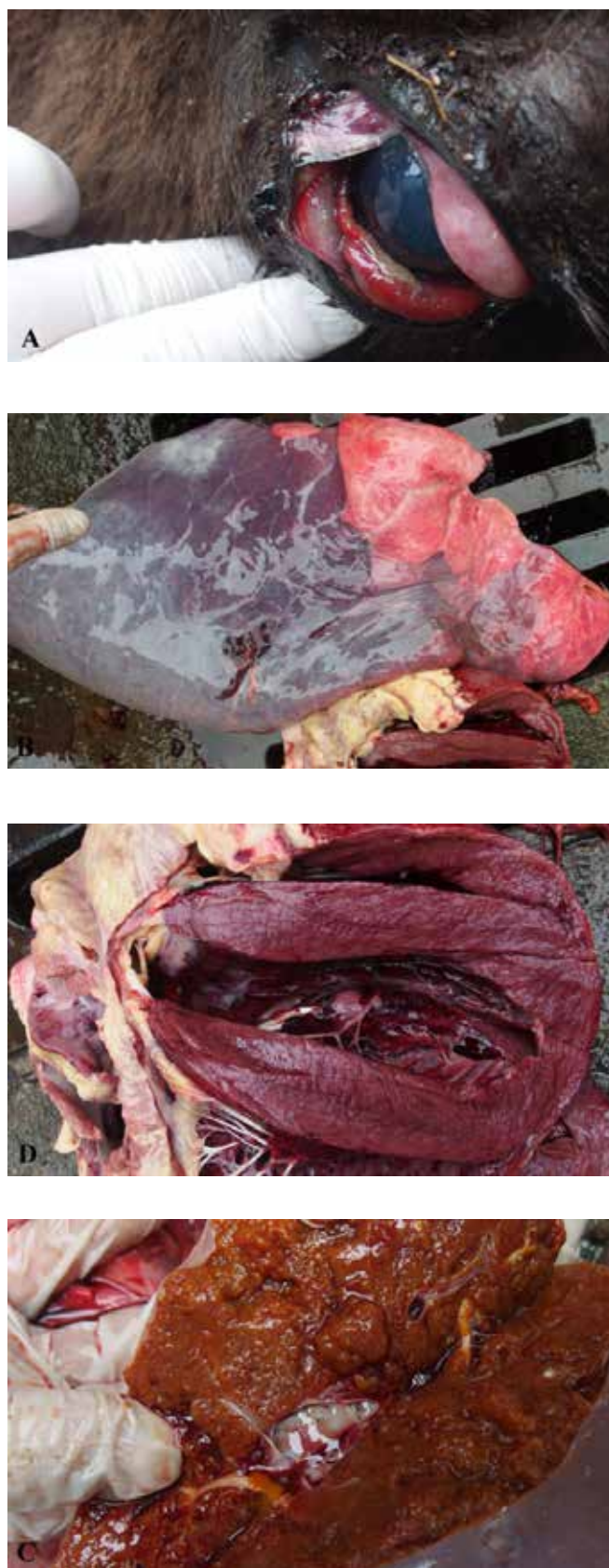


Fig. 1. Changes in cornea (A), lungs (B), liver (C) and hard muscle (D)

Fever, lack of appetite, spiking of hair, redness of the conjunctiva, serous and later purulent discharge from the nose, teeth grinding, ataxia, bloody diarrhea, depressed condition, inability to stand up, bedsores and subsequent death within 24-48 h were found in bison and big ruminants (gaurs and yak).

The pathology examination found mucopurulent exudates in the nasal cavities and bilateral graying of the cornea (Fig. 1 A). No damage to the mucosa of the buccal papillae and palate was observed in the oral cavity. Upon opening the trachea, petechial and ecchymosis hemorrhages, enlarged mediastinal lymph nodes were found. The lungs were stained blue and were sealed (Fig. 1 B). The liver was enlarged; the edges were rounded with a yellowish to orange color (Fig. 1 C). The gall bladder in some cases was the size of a small child's head. Mesenteric lymph nodes were not enlarged. The spleen was atrophic with necrosis, and upon resection there was no white pulp. On the epicardium and at the base of the heart multiple petechial and wider bleeding was visible, and the blood vessels were strongly injected with blood (Fig. 1 D).

Multiple petechial and more extensive longitudinal bleeding (Zebra stripes) was found in the mesentery of the small intestine (Fig. 2 A), the most severe in the first and middle sections of the small intestine (Fig. 2 B). There was no bleeding in the colon, and the intestinal content was of dark brown color. The kidneys were of dark red color (Fig 2 C), the urinary bladder was with urine and injected blood vessels. The rumen and abomasum did not appear to have any visible changes, but resection revealed reddened mucous membrane with a lot of petechial hemorrhages.

The samples obtained from diseased and dead animals were used to infect primary and permanent cell cultures. The best condition for the MCF virus growth was observed on the MDBK cell line after more than 21 passages. The visible cytopathic effect initially was formed 6-7 days after inoculation. The cytopathic effect was visible earlier with the increase in the number of passages. At 24th h after infection with the camel isolate, cell rounding and grapelike cluster formations were visible, the changes in the cell monolayer were augmented at 48th h and a large part of cells were detached in the maintenance medium at 72nd h (Fig 3. A, B and C). Large and small syncytia were observed from the bison and gaur isolates after 24th h. With the progression of sub-cultivation, the viral growth increased, CPE accelerated and after 48-60th h the number of

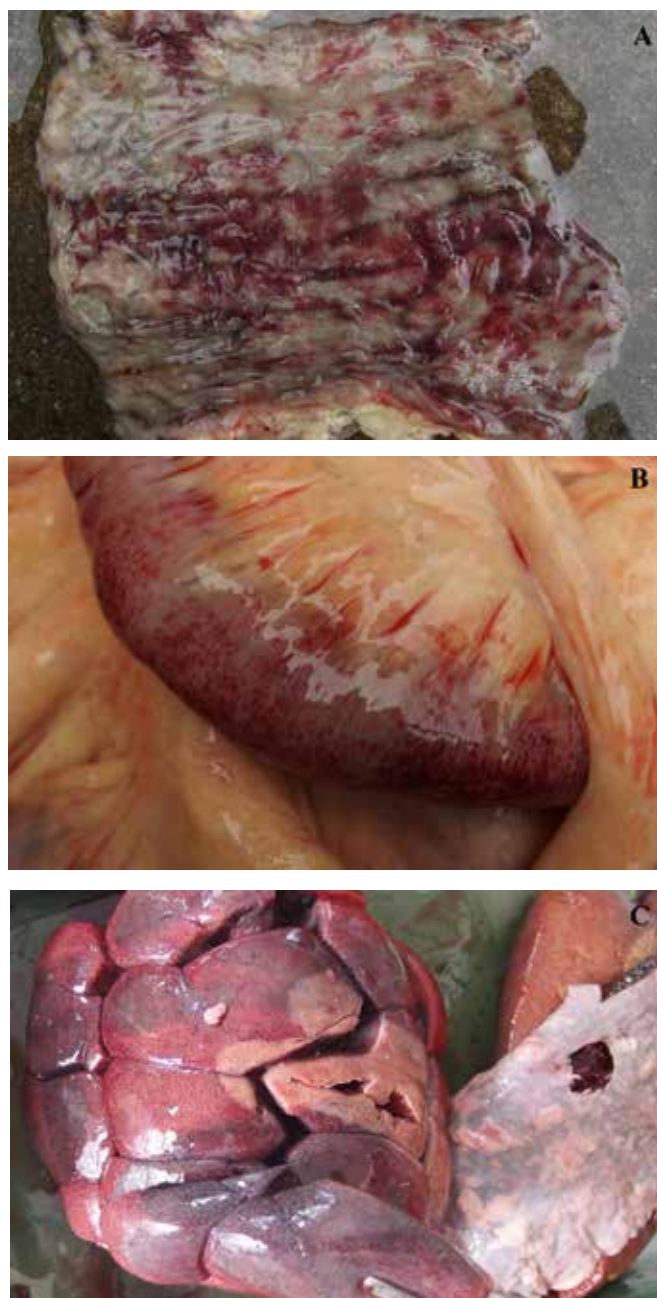


Fig. 2. Changes in small intestines (A and B) and kidney (C)

syncytia also increased and approximately 50% of the monolayer was affected. Full destruction of the cell monolayer was observed at 72nd h.

Viral titers of the isolated viruses on cell culture from gaur, camel, yak and two bison varied between $\log_{10}^{6.6}$ TCID₅₀/mL and $\log_{10}^{7.3}$ TCID₅₀/mL. The viral titer of the heterologous RNA paramyxovirus parainfluenza 3 (Pi-3) strain *Svetovrachene* was $\log_{10}^{5.33}$ TCID₅₀/mL. Viral titers decreased by 2 - 3 $\log_{10}$ after treatment of the viruses with 5-jod 2-desoxyuridine 5-iodo-2-deoxyuridine, while the titer of heterologous Pi-3 *Svetovrachene* strain did not change. CPE was not observed after treatment of all isolated viruses with

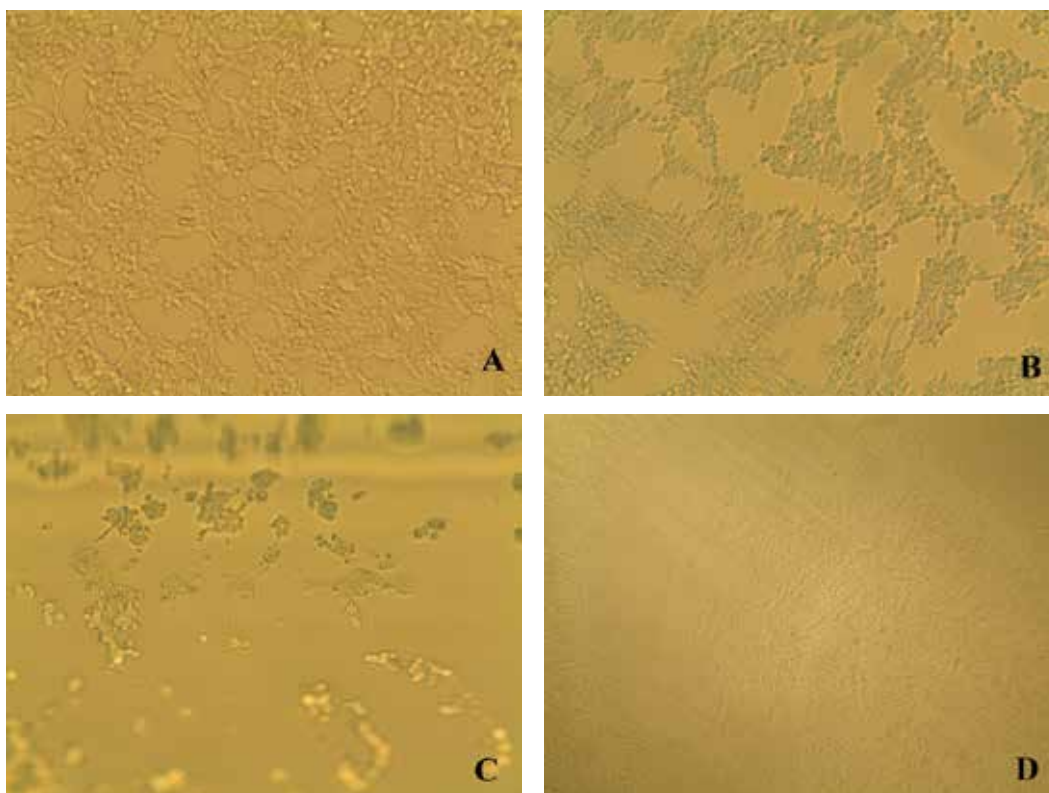


Fig. 3. Cytopathic effect of 21 passage after infection of MDBK cell culture with 10% lung suspension from camel with MCF clinical symptoms: A – 24th, B - 48th, C - 72nd hours after cell culture inoculation and D – MDBK negative control. Magnification 200X

20% ether and following inoculation of MDBK cell culture.

DNA was received from all native samples obtained from the investigated wild ruminants and cell cultures with visible CPE. The amplification products obtained by nested PCR with primers and procedures for OHV 2 after the first round of reaction were obtained amplicons with the size of 422 bp, and after the second round the size of 238 bp (Fig. 4). Isolated DNAs were not amplified by PCR with primers multiplying AIHV 1 for all isolates.

Thirty seven serum samples originated from 17 sheep, 13 Cameroon goats, 5 mouflons and two bison were investigated serologically by a competitive-inhibition ELISA for spread of MCF. In 21.4% of sheep sera, 30.7% of Cameroon goats sera, and 50% of bison sera antibodies against MCF were found. The total distribution of MCF antibodies in wild and domestic ruminants was 27%.

Discussion

Wild animals are a reservoir and source of MCF virus (Castro *et al.*, 1981). It has been proved that the infectious virus is spread by young newborn calves up to 3 months old (Plowright, 1968; Castro *et al.*, 1984). In our investigation the ani-

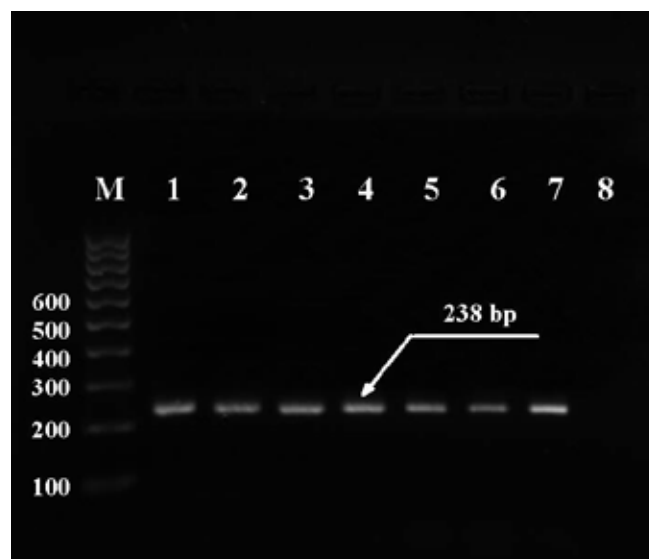


Fig. 4. PCR proving the MCF virus in native and MDBK cell culture samples. M molecular size marker 100 bp. MCF DNA originated from cell culture: Line 1- buffy coat bison, Line 2 – spleen gaur Line 3 – lungs camel, Line 4 – spleen hippopotamus, and native samples: Line 5 – lungs camel, Line 6 – spleen bison. Line 7 – positive control Germany, Line 8 – negative control none infected cell culture MDBK

mals were at different age. All animals investigated by us with clinical symptoms of MCF had been in direct contact with sheep and in indirect contact with wild life blackbuck antelope (*Antelope cervicapra*), antelope kana and capricorn (Hristov and Peshev, 2014). Papers on the epizootics of MCF in zoo parks are rare (Castro *et al.*, 1981; Campolo *et al.*, 2008). Our study proved that in Sofia zoo there is MCF epizooty. This assumption was confirmed by an ELISA study, in which different types of animals with antibodies against MCF were found.

Plowright *et al.* (1963), Plowright (1968), Castro *et al.* (1982) have found large-sized syncytium and inclusion bodies in bovine embryonic testis cells and bovine fetal kidney of isolates originating from an Indian gaur and Greater kudu with typical clinical signs of MCF. Likewise, we have observed growth of infected MDBK cell culture with samples from organ suspensions from gaur. The observed CPE in MDBK cell culture inoculated with organ suspension from bison with typical signs of MCF (Hristov and Peshev, 2015) is different from camel isolates. The different type of CPE cannot be explained with different titers of virus isolates, which varied between $\log 10^{6.6}$ for gaur to $\log 10^{7.3}$ for bison and $\log 10^{7.0}$ TCID₅₀/mL for camel. Most probably, the viral isolates from bison and gaur are genetically different than camel isolates.

Reduction of the viral titers with 2 and 3 log₁₀ after application of biochemical methods – iodine-deoxyuridine treatment for proving viral DNA on the cell culture isolates is the evidence that the investigated isolates are DNA viruses. It is proved by the results obtained by classical and nested PCR. Till now data on the isolation of OHV 2 virus was missing. The negative results from PCR specially designed for AIHV used in this study confirm that the cell culture MCF isolates in our investigation are OHV 2 viruses. Additional genetic investigations are needed to confirm the type of these isolates.

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Seroprevalence of Cytomegalovirus in the North-Eastern Bulgarian Population, 2003-2015

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Abstract

The prevalence of cytomegalovirus (CMV) infections is between 50% and 85% in adults in different parts of the world. Its epidemiology varies on socioeconomic and age groups. The present retrospective study has been performed to determine the seroprevalence of CMV among the population in North-Eastern Bulgaria. For the period 2003–2015, the prevalence of individuals with antibodies to CMV was estimated, using indirect enzyme-linked immunosorbent assay (ELISA) to detect virus-specific IgG and IgM. The population sample included 7879 randomly chosen hospitalized patients of both sexes and different ages. The total seroprevalence of CMV was determined to be 78.4% (CI 95% 77.5; 79.3), and the relative proportion of acute CMV infections 22.9% (CI 95% 21.9; 23.7). The proportion of CMV IgG and IgM by sex and by age was also analysed. The results of the study reveal that CMV infection is highly prevalent among the population and occurs mainly in the first years of life.

Key words: Cytomegalovirus (CMV), seroprevalence, anti-CMV IgM, anti-CMV IgG.

Резюме

Цитомегаловирусната инфекция е широко разпространена и в различни области на света е между 50% и 85% според наличието на специфични антитела във възрастната популация. Епидемиологичното разпространение на цитомегаловирус (CMV) варира в зависимост от възрастта и социално-икономическия статус на населението. Настоящото ретроспективно сероепидемиологично проучване има за цел да уточни серопревалентността спрямо CMV в общата популация в Североизточна България и честотата на първичното заразяване в зависимост от възрастта. За периода 2003 – 2015 г. беше определяно наличието на специфични anti CMV антитела от IgG и IgM тип в единична серумна проба и стандартизирана ELISA. Изследвани са 7879 пациенти от двата пола, разпределени във възрастови групи. Статистическият анализ беше направен с помощта на McCallum Layton calculators (www.mccallum-layton.co.uk) and Social Science Statistics (www.socscistatistics.com). В нашето проучване средната серопревалентност на общата популация е 78,4 %. Относителният дял на острите инфекции е 22.9 %. Според нашите данни CMV-инфекцията е широко разпространена и заразяването става най-често в ранното детство.

Introduction

Cytomegalovirus (CMV) is an ubiquitous large enveloped dsDNA β -herpesvirus with no known seasonal predominant distribution and with a prevalence that ranges between 50% and 85% of adults (Staras *et al.*, 2006; Bate *et al.*, 2010). The epidemiology of CMV varies in different regions of

the world and in different socioeconomic and age groups (Chandler *et al.*, 1985; Fowler *et al.*, 1993). According to previous data in North-Eastern Bulgaria, people over 15 years of age are infected in 85%, including women of childbearing age, and the average seroprevalence is 69.8% (Ivanova, 2007)

CMV infections are most often asymptomatic

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or with nonspecific presentations in healthy population, but when symptomatic, can lead to a syndrome resembling infectious mononucleosis or acute hepatitis. The virus is excreted through all body fluids, and the most common route of transmission is close or sexual contact, although transmission can also occur through breast milk, solid organ transplant or blood transfusions (Ross and Boppana, 2005; Stagno and Britt, 2006). CMV primary infection occurs mostly in childhood and adolescence, but primary infections can also be observed in adults (Stagno and Britt, 2006). The virus establishes lifelong latency in monocytes and lymphocytes after the primary infection (Schrier *et al.*, 1985).

The infection is important and dangerous in certain risk groups such as immunocompromised patients and pregnant women. In immunocompromised individuals, CMV infection, reactivation or re-infection is the leading cause of morbidity and mortality, especially in association with transplants, haemodialysis, cancer, immunosuppressive therapy and infection with human immunodeficiency virus (HIV), as well (Pass, 2001).

In primary maternal infection, the probability of transmission of CMV to the fetus is approximately 30% to 40%. In women with pre-existing CMV immunity during pregnancy the probability of fetal CMV transmission decreases to approximately 0.5% to 1.4% (Stagno *et al.*, 1982; Schleiss, 2008) and congenital infections in such cases can be less severe (Fowler *et al.*, 1992). According to the data, the overall birth prevalence of congenital CMV infection is reported to be 0.64%, but varies considerably among different study populations from 0.3% to 2.3% of all live births (Kenneson and Cannon, 2007). Congenital CMV infection is defined by either the detection of CMV-DNA or anti-CMV IgM in infant body fluids or sera within the first 21 days post-partum (Mosca and Pugni, 2007). Approximately 10% to 15% of infants with congenital CMV infection are symptomatic at birth, with manifestations including growth retardation, prolonged jaundice, thrombocytopenic purpura, hepatosplenomegaly, microcephaly, CNS calcifications and abnormalities, and retinitis. The risk of long term neurodevelopmental disabilities is high in these children and include microcephaly, hearing loss, motor deficits, cerebral palsy, mental retardation, seizures, ocular abnormalities and learning disabilities (Kylat *et al.*, 2006; Sharon and Schleiss, 2007).

Little is known about the seroprevalence and the relative proportion of CMV infections in Bul-

garia as national and regional trends in CMV infection have not been examined recently. The current work aimed to assess the seroprevalence of CMV and to determine the relative proportion of recent CMV infections in different age groups among the North-Eastern Bulgarian population.

Material and methods

Study population

From January 2003 to December 2015, a total of 7879 individuals of both sexes, distributed in 12 age groups: 0-6 months, 7 months-1 year, 2 - 5, 6 - 10, 11 - 15, 16 - 20, 21 - 25, 26 - 30, 31 - 40, 41 - 50, 51 - 60, and ≥ 61 years were studied in single serum samples for the presence of CMV-specific antibodies.

Method

Commercial ELISA test kits for detection of specific anti CMV IgM and IgG antibodies (EU-ROIMMUN – Germany, VIRCELL – Spain, Dia Pro – Italy, Adaltis – Italy) were performed according to the producer's recommendations. Anti-CMV IgM positive samples were considered as acute/recent infections.

Statistical analyses

The proportion of CMV seropositive individuals and their corresponding confidence intervals (CI), chi-squared distributions and p-values were calculated with McCallum Layton calculators and Social Science Statistics. A p-value < 0.05 was considered significant for all statistical tests.

Results

In a total of 7879 individuals, 6176 had IgG antibodies to CMV (78.4%, 95% CI: 77.5; 79.3) and 1703 were seronegative (21.6%, 95% CI: 20.7%; 22.5). Among the male participants, 3224 of 4220 (76.4%, 95% CI: 75.1; 77.7) were CMV IgG-positive and among the female participants 2952 of 3659 (80.7%, 95% CI: 79.4; 82.0) were CMV IgG-positives (Table 1). This difference in the proportions of seropositive males and females was statistically significant with chi-square of 21.2 and p-value of < 0.001 .

By distribution of CMV IgG-positive individuals (male and female) by age, 82.9% (95% CI: 79.9; 85.9) of children in the youngest age group (0 – 6 months) were shown to have antibodies (Fig. 1). It is consistent with the level of anti CMV IgG positivity (84.5% - (95% CI: 82.7; 86.3) found in women of childbearing age from the same population (Table 1), and most of them probably are transplacental. The percentage of seropositive individu-

Table 1. Individuals IgG-positive for cytomegalovirus, distributed by sex, 2003-2015

	Study participants (N)	N CMV-IgG positive	% CMV-IgG positive	95% confidence interval
Male	4220	3224	76.4	75.1 - 77.7
Female	3659 (1612*)	2952 (1362*)	80.7 (84.5*)	79.4 - 82.0 (82.7 - 86.3*)
Total	7879	6176	78.4	77.5 - 79.3

*Women of childbearing age 15-49 years (according WHO classification)

als then decreased and was similar in age groups of 7 months -1 year-old and 2 - 5 years-old with 53.7% (95% CI: 48.1; 59.3) and 52.6% (95% CI: 49.4; 55.8), respectively. Subsequently the proportion of seropositive individuals progressively increased with age, reaching 62.8% (95% CI: 58.9; 66.7) in the age group of 6 – 10 years-olds, 69.4% (95% CI: 65.4; 73.4) in the 11 – 15 years-old, 70.8% (95% CI: 66.8; 74.8) in the 16 – 20 years-old, 78.5% (95% CI: 74.8; 82.2) in the 21 – 25 years-old, 83.8% (95% CI: 80.6; 87.0) in the 26 – 30 years-old, 85.4% (95% CI: 83.2; 87.6) in the 31 – 40 years-old, 92.2% (95% CI: 90.2; 94.2) in the 41 – 50 years-old, 92.5% (95% CI: 90.6; 94.4) in the 51 – 60 years-old and 94.9% (95% CI: 93.5; 96.3) in the oldest group (≥ 61 years). The proportion of males and females with CMV IgG in each age group is shown in Figure 1.

By distribution of CMV IgM-positive individuals (male and female) by age, 20.5% (95% CI:

17.3; 23.7) of children in the youngest age group (0 – 6 months) were shown to have antibodies. The percentage of CMV IgM-positive individuals was 36.7% (95% CI: 31.3; 42.1) in the 7 months -1 year-old, 38.4% (95% CI: 35.3; 41.5) in the 2 - 5 years-old and then progressively decreased with age, reaching 30.8% (95% CI: 27.1; 34.5) in the age group of 6 – 10 years-olds, 26.2% (95% CI: 22.4; 30.1) in the 11 – 15 years-old, 28.7% (95% CI: 24.8; 32.7) in the 16 – 20 years-old, 21.5% (95% CI: 17.8; 25.2) in the 21 – 25 years-old, 21.1% (95% CI: 17.6; 24.7) in the 26 – 30 years-old, 19.6% (95% CI: 17.1; 22.1) in the 31 – 40 years-old, 16% (95% CI: 13.3; 18.7) in the 41 – 50 years-old, 13% (95% CI: 10.6; 15.4) in the 51 – 60 years-old and 12.8% (95% CI: 10.7; 14.9) in the oldest group (≥ 61 years). The proportion of males and females with CMV IgM in each age group is shown in Figure2.

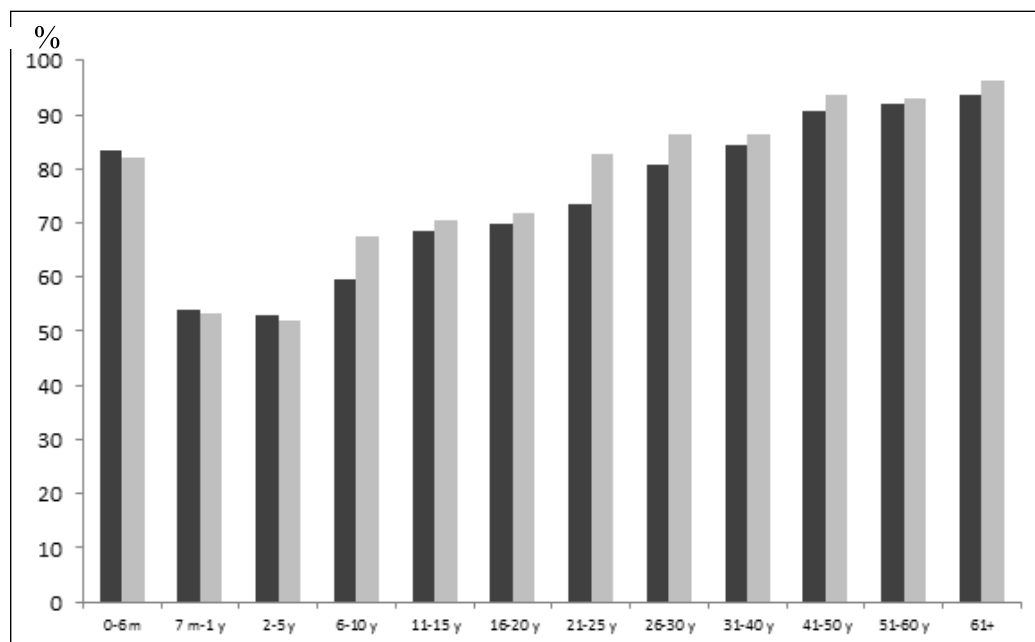


Fig. 1. Proportion of individuals IgG-positive for Cytomegalovirus, by sex (black – male and grey – female) and age, 2003-2015 (N=7879)

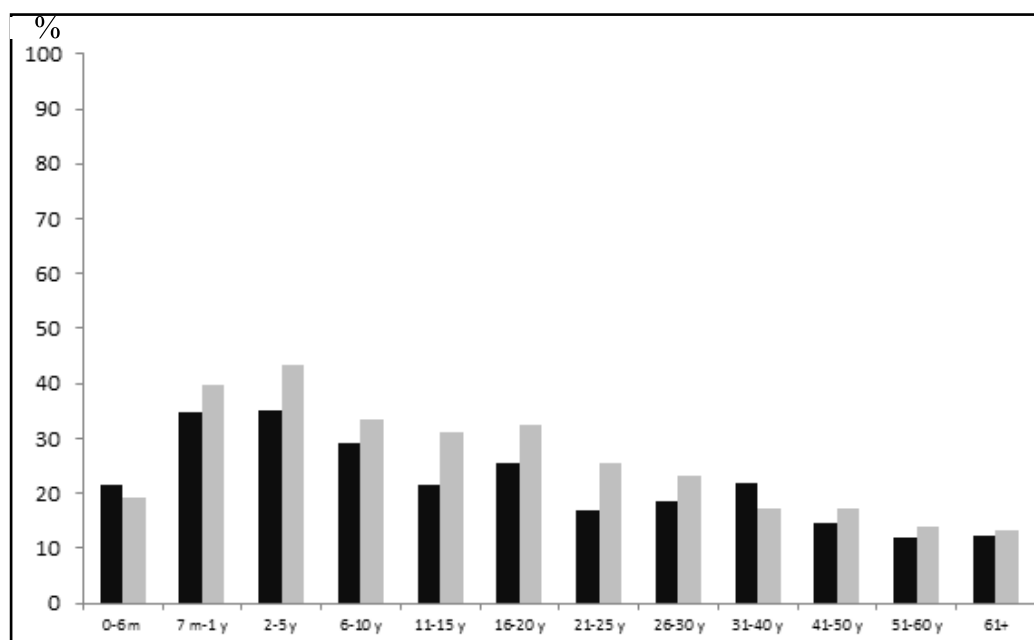


Fig. 2. Proportion of individuals IgM-positive for Cytomegalovirus, by sex (black – male and grey – female) and age, 2003-2015 (N=7879)

Discussion

The results of this study indicate that CMV infection is highly prevalent among the population (78.4%, 95% CI: 77.5; 79.3), similarly to reported results about other countries (Takeda *et al.*, 2001; Kothari *et al.*, 2002; Staras *et al.*, 2006). Such high seroprevalence may have profound effects with respect to immunocompromised individuals and maternal infections.

With respect to distribution by sex, women were found to be more infected than men, 80.7% (95% CI: 79.4; 82.0) and 76.4% (95% CI: 75.1; 77.7), respectively. The calculated risk ratio shows that females are exposed to a greater risk of CMV seropositivity by 6% (RR = 1.06, 95% CI: 1.03; 1.08) when compared to males. A possible explanation of this fact is that women more often become infected by sexual contact in addition to the contact route in the society, and are in close contact with the virus-containing secretions (urine, saliva) of their infected children. This horizontal route of transmission presents a risk to young mothers, pregnant women and those with occupations associated with exposure to children, such as teachers and day careworkers (Staras *et al.*, 2008). To support this, the study showed that 32.4% of the 16 – 20-year-old young women and 25.6% of the 21 – 25-year-old show evidence of acute infection. According to Italian and Brazilian authors, due to the high CMV seroprevalence in women of childbearing age, actually transmission in previously seropositive women through reactivation or re-in-

fection happens more often and is responsible for most congenital CMV infections (Barbi *et al.*, 2006; de Vries *et al.*, 2013). According to our data about CMV seroprevalence in women of childbearing age, the total risk of congenital infection is still higher in cases of primary maternal infection. In our population, 15.5% of women of childbearing age were found to be susceptible to primary CMV infection, which, combined with the fact that in this case the risk of transmission to the newborn is 40% (Stagno and Britt, 2006), gives a total population risk of 6.2%. Assuming that the risk of congenital infection after reactivation of maternal CMV infection during pregnancy is around 1% (Stagno *et al.*, 1982) and taking into consideration that 84.5% of women of childbearing age in our population were CMV IgG-positive, the total risk of congenital CMV transmission for the population when the mother is immune, is 0.8%, which is approximately 8 fold lower than after the primary infection.

With respect to age-sex distribution, women showed higher seroprevalence than men in all age groups after age 5. The seroprevalence in women is considerably higher than in men in the age group 21 – 25 years old (82.9% versus 73.6%, $p=0.01$). After this age group, the seroprevalence values for both sexes converge, but remain higher in women, although not statistically significant.

A tendency for prolonged presence of IgG antibodies in the 0 – 6 months age group (82.9%, 95% CI: 79.9; 85.9) was also observed, probably due to complementary postnatal infection. Our conclusion

is confirmed by a relatively high proportion of acute infections (IgM anti CMV positivity) in the 0 - 6 month's group (20.5%), which continues to grow in the next age groups - 7 - 12 months (36.7%) and 2 - 5 year-old (38.4%). Our data indicate that more than 1/3 of the children under 5 years of age acquire CMV infection. Anti CMV IgG seropositivity gradually increases in the older age groups. For our study population, more than 90% of people above 40-ies are infected.

According to our study, seroprevalence increased with age and concurrently the proportion of the acute infections decreased as a general tendency. A considerably high proportion – 25.9% (95% CI: 23.1; 28.7) of children in the youngest age groups (0 – 12 months) were shown to have IgM antibodies. The proportion of infants who become infected with CMV during the first year of life is directly related to the seroprevalence of women of reproductive age and to the proportion of mothers who breastfeed. In countries where breastfeeding is widely practiced and the majority of mothers are seropositive, for example in south Europe, some regions of Asia, Africa and Latin America, more than 50% of infants acquire CMV within the first year of life (Stagno *et al.*, 1982). The highest proportion (38.4%, 95% CI: 35.3; 41.5) of acute infections (anti-CMV IgM positive) was obtained in the 2 - 5 age group, when children are admitted in day care centers (de Mello *et al.*, 1996).

Conclusions

1. CMV infection is highly prevalent (78.4%) and has increased in comparison with a previous study (69.8%) (5) in the North-Eastern Bulgarian population, probably due to the more sensitive modern ELISA tests, and the larger number of tested individuals.
2. Primary CMV infection occurs mainly in the first 5 years of life.
3. Serological screening and viral load testing should be performed for immunocompromised patients and pregnant women or women who intend to become pregnant.
4. The main preventive measures against CMV infections and reinfections should be aimed at immunocompromised patients and women who intend to become pregnant, and women who work with children. To these individuals the exposure to CMV can be most detrimental and even fatal. They are the target groups for administration of a future effective vaccine.

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Seroprevalence of Epstein-Barr Virus in North-Eastern Bulgaria

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Abstract

Epstein-Barr Virus (EBV) is a widely spread member of the *Herpesviridae* family. Data show that in more than 90% of the adult population, specific anti-EBV can be found. In different Bulgarian studies the seroprevalence was set between 60% and 80%. The primary infection early in life is often asymptomatic or with nonspecific clinical symptoms, while in teenagers infectious mononucleosis develops. EBV is a proven agent of malignant, autoimmune and lymphoproliferative diseases, especially in immunocompromised patients. Tracking and analyzing the seroepidemiological status of the population in every region is of utmost importance. In our study, 5016 single serum samples from patients referred for EBV testing for various reasons were analyzed for the period 2010-2015. People aged between 1 month and 89 years old were divided into sixteen age groups. The samples were tested using indirect enzyme-linked immunosorbent assay (ELISA) for detection of anti-EBV (VCA) IgM and IgG. The proportion of EBV seropositive individuals and their corresponding confidence intervals (CI), chi-squared distributions and p-values were calculated. Our data indicated high, age-dependent EBV (VCA) IgG dissemination with 90% seroprevalence after age 26. Primary infections detected by anti-EBV (VCA) IgM occur in a bimodal model with peaks in age groups 1 – 5 and 11 – 20 years old.

Key words: Epstein-Barr virus (EBV), seroprevalence, anti-EBV (VCA) IgM, anti-EBV (VCA) IgG, infectious mononucleosis (IM).

Резюме

EBV е широко разпространен представител на семейство *Herpesviridae*. По литературните данни повече от 90% от възрастното население притежава специфични антитела срещу вируса. Според различни проучвания в България серопревалентността се движи между 60% и 80%. Първичната инфекция в ранното детство често е асимптомна или протича с неспецифична клинична симптоматика, докато в юношеска възраст се развиват симптоми на инфекциозна мононуклеоза. EBV е доказан причинител на малигнии, автоимунни и лимфопролиферативни заболявания, особено при имунокомпрометирани пациенти. Проследяването и анализирането на сероепидемиологичния статус на населението във всеки регион е от изключителна важност. Нашето проучване се основава на анализ на 5016 единични серумни проби от пациенти, насочени по различни причини за изследване на EBV-инфекцията към Лаборатория по клинична вирусология в УМБАЛ “Света Марина” – Варна в периода 2010 – 2015 г. Пациентите бяха разделени в 16 възрастови групи. Използвани са стандартизирани ELISA китове за доказване на специфични anti-EBV (VCA) IgM и IgG. Статистическият анализ беше направен с помощта на McCallum Layton calculators (www.mccallum-layton.co.uk) и Social Science Statistics (www.socscistatistics.com). Нашите данни показват високо, възрастово свързано разпространение на заразеността с EBV, според наличието на anti EBV (VCA) IgG. Серопревалентността след 26-годишна възраст е над 90%. Първичната инфекция според наличието на anti-EBV (VCA IgM) има бимодален модел на разпространение с пикове във възрастови групи 1 – 5 и 11 – 20 години.

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Introduction

Epstein-Barr Virus (EBV) is a widely spread member of the family Herpesviridae. The unique properties of the viruses of this family are their lifetime persistence in latent stage after primary infection and reactivation capability (Middleton, 1974; Tan *et al.*, 1999; Rickinson and Kieff, 2007). More than 90% of the adult population possesses specific anti-EBV (VCA) IgG, and according to various studies in Bulgaria, seroprevalence is 60-80% (Dundarov, 1998; Genova *et al.*, 2013). A correlation between the time of the primary infection and the socio-economic status of the population has been established (Henle *et al.*, 1979; Straus *et al.*, 1993). Previous studies in northeastern Bulgaria suggest that the infection occurs mostly by age 4-7 and marks a peak in the age group 15-22 (Ivanova, 2007). Most commonly a major mechanism of transmission is through kissing, but infection from blood transfusions, organ and bone marrow transplantation and sexual contact is also possible (Niederman *et al.*, 1976; Craig *et al.*, 1993; Cohen, 2000; Thomas *et al.*, 2006). The virus replicates in epithelial cells of the macro-organism and establishes latency in B-lymphocytes (Tan *et al.*, 1999; Rickinson and Kieff, 2007). Primary infection occurs as infectious mononucleosis (IM) mainly in adolescence and later. Infection during early childhood is more often asymptomatic or with non-specific symptoms (Dowd *et al.*, 2013; Straus *et al.*, 1993). In later life, EBV is associated with a variety of malignancies, autoimmune and lymphoproliferative disorders (Craig *et al.*, 1993; Alexander *et al.*, 2003; Magrath, 2012).

In recent years, the diagnosis of EBV infection is mainly performed by serological methods based on enzyme-linked immunosorbent assay (ELISA). Specific serological markers for acute infection are IgM and IgG antibodies of VCA (viral capsid antigen), anti-EA (D) IgG (early antigen) (Klutts *et al.*, 2009). Specific serological markers for past infection are anti-EBNA1 IgG and anti-EBV (VCA) IgG, which persist for life (Linde, 1996; Hess, 2004). The purpose of this study is a

retrospective analysis to clarify the seroepidemiological situation in northeastern Bulgaria and to define the time of primary infection with EBV.

Material and Methods

Tested population

In the period 2010 - 2015, 5016 hospitalized and outpatients were tested for various reasons, in a single serum sample in the Laboratory of Clinical Virology, University Hospital „St. Marina“ - Varna. Patients were divided into 16 age groups: 1 month-1 year old, 1-5 (years old), 6-10 (years old), 11-15 (years old), 16-20 (years old), 21-25 (years old), 26-30 (years old), 31-35 (years old), 36-40 (years old), 41-45 (years old), 46-50 (years old), 51-55 (years old), 56-60 (years old), 61-65 (years old), 66-70 (years old) and over 71. The average age of patients was 29.84 (SD±23.85). Minimal age was 1 month, the maximal – 89. In the studied sample, the proportion of male individuals was 54.7% (95% CI: 53.32% -56.08%), and females were 45.3% (95% CI: 43.92% - 46.68%) (Table. 1).

Methods

Standardized serological ELISA tests for the detection of specific anti-EBV (VCA) IgM (Anti-EBV-CA ELISA IgM, EUROIMMUNE- Germany) and IgG (Anti-EBV-CA ELISA IgG, EUROIMMUNE - Germany) were performed according to the producer's recommendations.

Statistical analysis

The proportion of EBV seropositive individuals and their corresponding confidence intervals (CI), chi-squared distributions and p-values were calculated with SPSS, v.19.0. A p-value <0.05 was considered significant for all statistical tests.

Results

The highest share of tested individuals was in the age group 1-5 (15.9%, 95% CI: 14.79% - 16.81%), possibly due to the need for more frequent consultation and laboratory diagnostics of atypical clinical manifestations in young children. The proportion of tested hospitalized patients was higher (86.04%, 95% CI: 85.08% -87%) compared

Table 1. Distribution of tested individuals by sex for the period 2010-2015

<i>Sex</i>	<i>Total number of tested individuals (N)</i>	<i>Proportion (95% CI)</i>
Men	2744	54.7% (53.32%-56.08%)
Women	2272	45.3% (43.92%-46.68%)
In total	5016	100%

to outpatients (13.96%, 95% CI: 13% -14.92%). This shows that tests for EBV are predominantly appointed in individuals during their hospital stay and in conjunction with concomitant unspecified illness.

Anti-EBV (VCA) IgG immune status:

From a total of 5016 participants in this study, 5012 were tested for the presence of anti-EBV (VCA) IgG (99.9%, 95% CI: 99.81% -99.99%) (Table. 2). Hospitalized patients were 86.1% (95% CI: 85.14% -87.06%) of the sample compared with outpatients (13.9%, 95% CI: 12.94% -14.86%).

4135 of the tested individuals showed positive results (82.5%) (95% CI: 81.45% -83.55%). We found differences in seroprevalence between outpatients (88.2% - 95% CI: 85.8% -90.6%) and hospitalized patients (81.6% - 95% CI: 80.44% - 82.76%).

The proportion of positive men (n = 2216) was 80.8% (95% CI: 79.33% -82.27% and the positive women were 1919 - 84.5% (95% CI: 83.01% -85.99%). The difference is statistically significant (p <0.05) (Cramer's V = 0.048, p <0.05).

There is a statistically significant relationship between the age of tested individuals and anti-EBV (VCA) IgG seropositivity, established with chi-squared test (p <0.01) and the fact that the strength of this relationship is weak (Cramer's V = 0.414,

p <0.01).

Anti-EBV (VCA) IgM antibody status:

For the presence of anti-EBV (VCA) IgM 4984 people were tested (99.4%, 95% CI: 99.19%-99.61%) (Table. 3). Hospitalized patients had a higher percentage (86.6%, 95% CI: 85.65% -87.55%) compared with the relative proportion (13.4%, 95% CI: 12.45% -14.35%) of outpatients. A positive result was confirmed in 876 (17.6% - 95% CI: 16.54%-18.66%). The proportion of positive outpatients was 15.8% (95% CI: 13.04% - 18.56%) and 17.8% (95% CI: 16.66% - 18.94%) for hospitalized patients. The positive males (n = 482) were 17.6% (95% CI: 16.8% - 19.02%) and females (n = 394) were 17.3% (95% CI: 15.74% - 18.86%). The difference was not statistically significant (p > 0.05).

The age of the tested people is associated with anti-EBV (VCA) IgM seroprevalence (p <0.05), and the power is not strong (Cramer's V = 0.307, p <0.01).

Discussion

According to the presence of anti-EBV (VCA) IgG in our study, 82.5% (95% CI: 81.45% -83.55%) of the population in Northeastern Bulgaria aged between 1 month and 89 years old shows positive evidence of infection with the EBV. In another study (Genova *et al.*, 2013), 79% of the population

Table 2. Age-related distribution of anti-EBV (VCA) IgG seroprevalence in northeastern Bulgaria in the period 2010-2015

Age	Persons (N)	Anti- EBV (VCA) IgG – seropositive	
		Anti-EBV (VCA) IgG seropositive by age	Proportion (95%CI)
under 1 yr.	314	215	68.5% (63.36%-73.64%)
1-5 yr.	793	404	50.9% (47.31%-54.29%)
6-10 yr.	408	308	75.5% (71.53%-79.87%)
11-15 yr.	316	262	82.9% (78.75%-87.05%)
16-20 yr.	355	294	82.8% (78.87%-86.73%)
21-25 yr.	265	237	89.4% (85.69%-93.11%)
26-30 yr.	280	261	93.2% (90.25%-96.15%)
31-35 yr.	320	298	93.1% (90.32%-95.88%)
36-40 yr.	300	286	95.3% (92.91%-97.69%)
41-45 yr.	226	216	95.6% (92.93%-98.27%)
46-50 yr.	231	215	93.1% (89.97%-96.43%)
51-55 yr.	223	208	93.3% (89.54%-96.26%)
56-60 yr.	248	235	94.8% (92.04%-97.56%)
61-65 yr.	237	223	94.1% (91.1%-97.1%)
66-70 yr.	231	223	96.5% (94.13%-98.87%)
Over 71 yr.	265	250	94.3% (91.15%-96.85%)
Total	5012	4135	82.5% (81.45%-83.55%)

Table 3. Age distribution of anti-EBV (VCA) IgM seropositivity in northeastern Bulgaria in the period 2010-2015

Age	Persons (N)	Anti-EBV (VCA) IgM – seropositive	
		Anti-EBV (VCA) IgM seropositive	Proportion (95%CI)
under 1 yr.	314	41	13.1% (9.37%-16.83%)
1-5 yr.	794	254	32.0% (28.7%-35.25%)
6-10 yr.	408	121	29.7%(25.07%-33.93%)
11-15yr.	314	100	31.8% (26.65%-36.95%)
16-20 yr.	354	126	35.6% (30.61%-40.59%)
21-25 yr.	263	42	16.0% (11.57%-20.43%)
26-30yr.	272	32	11.8% (7.97%-15.63%)
31-35 yr.	317	38	12.0% (8.42%-15.58%)
36-40 yr.	297	29	9.8% (6.42%-13.18%)
41-45 yr.	224	15	6.7% (3.43%-9.97%)
46-50 yr.	229	17	7.4% (3.95%-10.65%)
51-55 yr.	220	9	4.1% (1.77%-7.23%)
56-60 yr.	246	14	5.7% (2.8%-8.6%)
61-65 yr.	237	12	5.1% (2.3%-7.9%)
66-70 yr.	231	12	5.2% (2.34%-8.06%)
over 71 yr.	264	14	5.3% (2.91%-8.49%)
Total	4984	876	17.6% (16.54%-18.66%)

was found to be infected with the virus. A previous study for Northeastern Bulgaria determined an average prevalence of 60.83% with immunofluorescent test (Ivanova, 2007). Our data show increased prevalence compared to previous studies, which is possibly due to the higher number of investigated individuals and more sensitive methods. We affirm the prevalence of the virus, which is in line with the global tendency. In a study performed in Bangladesh and Taiwan across a wide age range population, the mean seroprevalence was 81.27% and 88.5% respectively (Haque *et al.*, 1996, Chao *et al.*, 2015). The data in our study confirm the global trends of increasing prevalence with age. Similarly, in Denmark, the proportion of people with antibodies increases from 15% in 1 year olds to 60-65% in the age group 4-6, reaching up to 95% in late adulthood (Hesse *et al.*, 1983). In the United States for the period 2003-2010, among the age group 6-19 the average seroprevalence was 66.5% and also was increasing with age (Dowd *et al.*, 2013).

The analysis of the age distribution of seroprevalence and primary infection with EBV in our survey shows 68.5% seropositivity in children under 1 year old. Anti-EBV (VCA) IgM antibodies were found in 13.1% (9.37% - 16.83%) of them. In this age range,

236 of the tested individuals were up to 6 months of age with 76.3% (95% CI: 72.17% - 82.38%) seropositivity to anti-EBV (VCA) IgG. For comparison, 92.4% (95% CI: 87.86% - 91.68%) of tested women of childbearing age defined by WHO (15-49 years old), were positive for anti-EBV (VCA) IgG, but according to literature, in pregnant women anti-EBV (VCA) IgG antibodies were detected in 94% to 100% (Dundarov, 1998; Crawford *et al.*, 2002). Therefore, in infancy, IgG antibodies probably are passively passed from the mother. This determines protection from infection and therefore the lowest share of early infected (12.7%) as shown by the test of anti-EBV (VCA) IgM. During early childhood (1-5 years) seroprevalence is the lowest - 50.9% (47.31% - 54.29%). Serological evidence of primary infection in this age group is 32.0% (28.7% - 35.25%). Seropositivity after this age group gradually increases and reaches 93.2% (90.25% -96.15%) in the age group 26-30, then continues to grow reaching 94.3% (91.15% - 96.85%) in age over 71. The highest proportion of anti-EBV (VCA) IgM seropositive people is observed in the age group 16-20 - 35.6% (30.61% -40.59%) and with age it gradually declines, reaching values between 12% and 4.1% in the age groups over 31.

The distribution of seropositivity by sex in our study shows a higher degree of infection in women (84.5%) compared to men (80.8%) ($p < 0.05$). These data do not correlate with the findings of Genova *et al.*, indicating male/female ratio 2:1 Genova *et al.*, 2013), but correlate with data from other studies (Biggar *et al.*, 1981; Crawford *et al.*, 2006; Higgins *et al.*, 2007; Dowd *et al.*, 2013), where the proportion of women was also higher.

According to available literature, EBV infection begins in childhood or adolescence and identified risk factors for the duration of infection are mainly socio-economic (Cecilia *et al.*, 2004). The analysis of our data from Northeastern Bulgaria shows that primary infection with EBV is attributed to early childhood and pre-school and school period. About 1/3 of children are infected by age 5, possibly with nonspecific clinical signs, and only the awareness of clinicians in our region refers patients to specific serological testing for EBV. Our data indicate a bimodal model of primary infection with a higher proportion in the age groups 1-5 and 11-20. According to earlier studies for the same region, the peaks of infections are observed in age groups 4-7 and 19-22 (Ivanova, 2007). Infection in early childhood is associated with the main route of transmission of the virus through oropharyngeal secretions in the family or in organized children's collectives. Our study did not test the impact of the household income, education level or other factors which could also have importance in determining the risk of infection in this age group. We think the higher prevalence is due mainly to the wide distribution of EBV and periodic transmission of the virus with oropharyngeal secretions from asymptomatic carriers. Periodic transmission of the virus in saliva was found in 15% - 20% of healthy seropositive youngsters and in immunosuppressed patients (Straus *et al.*, 1993, Ling *et al.*, 2003a). The second prominent peak during adolescence is due to the addition of sexual contact and the risk is mainly determined by the number of sexual partners. Accordingly, the virus is often confirmed in genital secretions. In surveys amongst young people not using protection during sexual intercourse, a higher incidence of infection was found, but further studies are needed, since the infection through kissing cannot be ignored (Crawford *et al.*, 2002; Higgins *et al.*, 2007). The high frequency of infection during adolescence suggests more frequent occurrence of infectious mononucleosis in this period.

Conclusion

In our survey we found:

1. EBV infection is highly prevalent in Northeast Bulgaria (82.5% - 95% CI: 81.45%-83.55%) and increased in comparison with a previous study, probably due to the more sensitive modern ELISA tests, and the larger number of tested individuals.
2. EBV infection is highly prevalent in women (84.5%) compared to men (80.8%) ($p < 0.05$).
3. Our data indicate a bimodal model of primary infection with a higher proportion in the age groups 1 - 5 and 11 - 20.
4. Serological screening and viral load testing should be performed for immunocompromised patients and patients with different malignancies with respect to reactivation and/or reinfection with possible fatal complication.

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Investigation of the Antiviral and Antibacterial Potential of Coelomic Fluid from Some Earthworm Species (*Oligochaeta: Lumbricidae*)

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Abstract

The coelomic fluid of four earthworm species, *Aporrectodea rosea*, *Eisenia fetida*, *Lumbricus terrestris* and *Octolasion lacteum*, was investigated against Gram-negative bacteria strains: *Aeromonas hydrophila*, *Pseudomonas fluorescens* and *Pseudomonas luteola* and human pathogen Herpes simplex virus type 2 (genital herpes). The research revealed that only studied material from *Eisenia fetida* acted selectively on pathogenic bacteria. The greatest growth-inhibiting effect was observed on *Aeromonas hydrophila*. No antiviral potential was demonstrated against Herpes simplex virus. The metabolic components of the coelomic fluid have a strong cytolytic effect, but lack any activity against the tested virus pathogen.

Key words: coelomic fluid, *Pseudomonas*, *Aeromonas*, Herpes simplex virus, earthworms, Lumbricidae

Резюме

Влиянието на целомната течност от четири вида дъждовни червеи: *Aporrectodea rosea*, *Eisenia fetida*, *Lumbricus terrestris* и *Octolasion lacteum* е изследвано върху някои Грамотрицателни бактерии: *Aeromonas hydrophila*, *Pseudomonas fluorescens* и *Pseudomonas luteola*, както и спрямо Herpes simplex virus тип 2 (генитален херпес). Получените резултати показват, че само *Eisenia fetida* има изразен антибактериален ефект върху изследваните микроорганизми като ефекта е най-силен спрямо *Aeromonas hydrophila*. Метаболитните компоненти на целомната течност имат силна цитолитична активност при въздействие на използваната от нас клетъчна линия, но не повлияват репликацията на човешкият херпесен вирус тип 2.

Introduction

Earthworms live in an environment abundant in pathogens. Some of those pathogens are bacteria and viruses. Earthworms lack true antibodies, hence an adaptive immune response and the coelomic fluid act as an efficient innate immune system to defend them against invading pathogens (Liu *et al.*, 2004).

The coelomic fluid of the earthworm *Eisenia fetida* (*Oligochaeta: Lumbricidae*), containing more than 40 proteins, exhibits several biological effects as follows: cytolytic, proteolytic, hemolytic, hemagglutinating, tumorstatic, mitogenic, and bacteriostatic activities (Lange *et al.*, 1997). The cytolytic components secreted by coelomocytes into the coelomic cavity are of particular interest in view of

their potential clinical applications. They include factors displaying hemolytic activity accompanied by antibacterial/bacteriostatic effects against pathogenic soil bacteria (Roch *et al.*, 1979; Valembois *et al.*, 1982; Roch *et al.*, 1992). The second group includes factors exerting lytic activity towards other cell types, particularly tumor cells (Bilej *et al.*, 2002).

Aeromonas hydrophila can cause gastroenteritis in humans, occurring mostly in young children and people with compromised immune systems. These bacteria are linked to two types of gastroenteritis. The first type is rice-water diarrhea. The other type is dysenteric gastroenteritis, which causes loose stools filled with blood and mucus. *Aeromonas hydrophila* also causes diseases such as myonecrosis and eczema in people with com-

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promised or suppressed immune systems (Janda and Abbott, 1998). In very rare cases it can cause necrotizing fasciitis (Minnaganti *et al.*, 2000). *Aeromonas hydrophila* is proved to be an earthworm pathogenic bacterium, especially to *Eisenia fetida* (Lassegues *et al.*, 1989). *Pseudomonas luteola* is a saprophyte. It is an opportunistic pathogen that particularly affects patients with health disorders. Most reported cases showed septicemia, meningitis, endocarditis or peritonitis (Chihab *et al.*, 2004). Gershman *et al.* (2008) reported that *Pseudomonas fluorescens* is an unusual cause of disease in humans, and usually affects patients with compromised immune systems (e.g., patients on cancer treatment).

Herpes simplex virus type 2 (HSV-2) is a sexually transmitted pathogen that infects more than 500 million people worldwide and causes an estimated 23 million new infections each year. It is predominantly associated with genital infection (Johnston *et al.*, 2011).

The aim of our study was to investigate the antiviral and antibacterial potential of the coelomic fluid from earthworms *Aporrectodea rosea*, *Eisenia fetida*, *Lumbricus terrestris* and *Octolasion lacteum* against Gram-negative bacteria strains: *Aeromonas hydrophila*, *Pseudomonas fluorescens* and *Pseudomonas luteola*, and the human pathogen Herpes simplex virus type 2 (genital herpes).

Materials and methods

Collection area

The field investigations were carried out during the spring of 2015 in Leached Vertisols (Bozhurishte, Bulgaria). Earthworms were collected by digging and hand-sorting. Four lumbricid species were found: *Aporrectodea rosea* (Savigny, 1826), *Eisenia fetida* (Savigny, 1826), *Lumbricus terrestris* (Linnaeus, 1758) and *Octolasion lacteum* (Örley, 1881).

Coelomic fluid sampling

The earthworms were washed in running distilled water. They were placed on filter paper to remove excess water droplets and were taken into a glass petri dish. The lumbricids were induced to extrude coelomic fluid through the epidermal dorsal pores by 5 V electric stimulation. Then the stimulated earthworms in petri dishes were rinsed with pH 6.8 phosphate-buffered saline (PBS). The collected coelomic fluid containing PBS was centrifuged at 5000 rpm for 10 minutes to sediment coelomocytes and particulate materials. The supernatant was carefully removed and filter sterilized

through 0.2 µm (pore size) syringe filter. The filtrate of the coelomic fluid that was free from any suspended cells or coelomocytes (CF) was stored in aliquots at -20°C for subsequent use.

Antibacterial assay

The strains used for determining antimicrobial activity included soil pathogen bacteria: *Aeromonas hydrophila*, *Pseudomonas fluorescens* and *Pseudomonas luteola* obtained from the Department of Soil Microbiology, Institute of Soil Science, Agrotechnologies and Plant Protection “N. Poushkarov”, Sofia, Bulgaria. Bacterial cells were inoculated in nutrient agar with a final concentration of 10⁸ colony-forming units/mL on a 90-mm Petri dish. After the top agar hardened, it was placed on sterilized blotting paper (about 7 mm in diameter), free from any antibacterial activity, and was impregnated with 20 µL of the earthworm coelomic fluid to be tested and placed on agar dishes inoculated with one bacterial strain. The dishes were incubated overnight at 28°C. Control tests were performed with papers impregnated with sterile saline solution (0.9% NaCl). Antimicrobial activity was determined by observing the zone of suppression of bacterial growth around the 7-mm papers.

Cells and viruses

In the study MDBK (Madine and Darby bovine kidney) cells were used, grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal calf serum (FCS) (with Gentamycin 8 µg/ml and 10 mM HEPES buffer). The maintenance medium contained fetal calf serum with reduced concentration of 2.5%.

This study employed the BA strain of HSV-2. The virus was propagated in MDBK cells and stored at -70°C until used. The virus titer was determined by cytopathic effect (CPE) assay using the method of Reed and Muench (Reed and Muench, 1938) and plaque assay (Dulbecco, 1952) in MDBK.

Cell toxicity

The coelomic fluid of the collected earthworm species was twice diluted in maintenance media just before the experiments (*ex tempore*). The concentration of the coelomic fluid is presented as a percent (%) in each dilution.

Cell toxicity was monitored by determining the effect of the studied materials from the explored earthworm species on cell morphology and cell viability. The morphology of the cells was inspected daily and observed for microscopically detectable alterations, i.e. loss of monolayer, rounding, shrinking of cells, granulation, vacuolisation in the cytoplasm. Cell viability was determined by a colorimetric

metric method (MTT assay) (Mosmann, 1983). It is based on the reduction of MTT [3-(4, 5-dimethylthiazol-2ol)-2,5diphenyltetrazolium bromide), Sigma Chem., Co. St. Louis, USA], by the mitochondrial enzyme succinate dehydrogenase of the viable cells, which develops a formazan blue colour product. Confluent monolayers of MDBK cells in 96-well plates were overlaid with 0.1 ml/well maintenance medium supplemented with 2.5% FCS (with Gentamycin 8 µg/ml and 10 mM HEPES buffer), 0.1 ml/well of the dilutions of the obtained coelomic fluid (3 well/dilution) and were incubated at 37°C for 48 h. Minimum 3 wells in the plate were used as controls and were not inoculated (only maintenance medium was added). On the second day, 20 µl of MTT (5 mg/ml in phosphate buffered saline (PBS)) was added to each cell; the monolayers were incubated for 3–4 h at 37°C. The resulting formazan precipitate was dissolved in dimethyl sulfoxide (DMSO). After a few minutes at room temperature to ensure that all crystals were dissolved, the optical densities (OD) were determined by Multiscan MX plate reader with a 540 nm. The percentage of viable treated cells was calculated in relation to the untreated controls:

$[(OD_{exp.})/(OD_{cell\ control})] \times 100$, where $(OD_{exp.})$ and $(OD_{cell\ control})$ indicate the absorbencies of the test sample and the cell control, respectively. The 50% cytotoxicity concentration (CC_{50}) was determined as the test compound concentration required for reduction of cell viability by 50%.

Virucidal assay

The direct virus inactivating effect of the studied materials was tested by a direct contact assay. Undiluted stock virus suspensions were treated with equal volumes of the coelomic fluid MNC (maximal nontoxic concentration), prepared in DMEM supplemented with 2.5% FCS and incubated at 37°C for 5', 15', 30', 60', 120', 240' and 360' (in an eppendorf tube). Compound free DMEM, supplemented with 2.5% FCS, was used for viral control. At the end of each time interval, the control and the treated viruses were frozen, and the difference in the biological activities between them was determined on the basis of infectivity. The surviving infectious virus titers were determined using the method of Reed and Muench (Reed and Muench, 1938).

Cell protection assay

A modification of an MTT assay developed for screening anti-HSV compounds (Takeuchi *et al.*, 1991) was used. Confluent monolayers in 96-well plates were overlaid with 0.1 ml/well of virus

suspension (low multiplicity of infection (MOI) 100 TCID₅₀ (tissue culture infectious dose)/well, 0.0039 PFU (plaque forming unit)/cell). The plates were incubated for 1 hour at 37°C for virus adsorption and dilutions of the obtained coelomic fluid. Maintenance media were added for virus control. Uninoculated cells were used for cell control. On the fifth day, 20 µl of MTT (5 mg/ml in PBS) was added to each well and the monolayers were incubated for 3–4 h at 37°C. The medium with MTT was removed and the resulting formazan precipitate was dissolved in DMSO. The extinctions were determined at $\lambda=540$ nm. The percentage of protection was calculated by the following formula: $[(OD_{exp.})-(OD_{virus\ control})]/(OD_{cell\ control})-(OD_{virus\ control})] \times 100$, where $(OD_{exp.})$, $(OD_{virus\ control})$, and $(OD_{cell\ control})$ indicate the absorbencies of the test sample, the virus control and the cell control, respectively.

Results

Cellular toxicity

The coelomic fluid obtained from the collected earthworm species was applied in concentrations ranging from 0.39 % to 25 %. Coelomic fluid from three of the lumbricid species *Aporrectodea rosea* (Savigny, 1826), *Lumbricus terrestris* (Linnaeus, 1758) and *Octolasion lacteum* (Örley, 1881) showed no visual change in the cell monolayer integrity (daily microscopic observation) or any difference in viability of the treated cells and controls, even in the lower dilutions (25%). Unlike them, the studied material from *Eisenia fetida* (Savigny, 1826) behaved in a different way (Fig. 1).

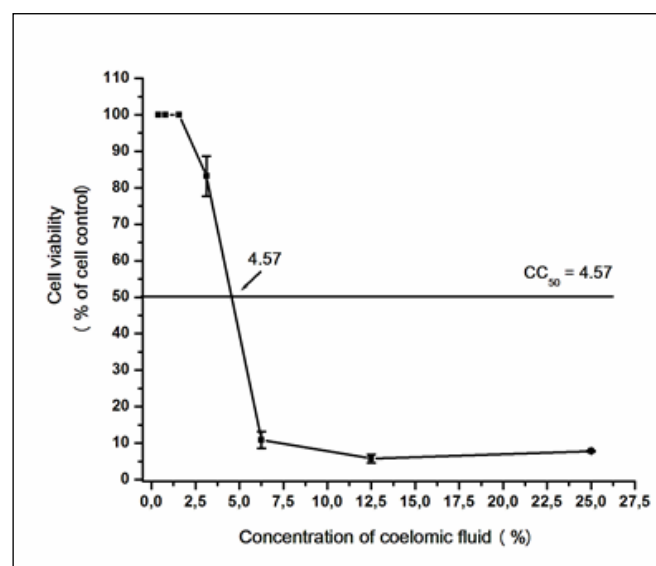


Fig. 1. Toxicity of coelomic fluid from *Eisenia fetida* against MDBK cell line

It shows toxicity against MDBK cell line in a dose-dependent manner. According to the MTT test, cell viability was very low in a concentration range between 25 % and 6.25 %. Cells reached 50 % viability (cytotoxic concentration 50 (CC₅₀) at a concentration of 4.57% of coelomic fluid.

Antiviral activity of coelomic fluid

Virucidal activity

For more complete understanding, we tested the direct virus inactivating effect against HSV-2, strain BA of the obtained coelomic fluid from all collected earthworm species by direct contact assay. Undiluted stock virus suspensions were treated with equal volumes of studied materials in MNC for different time intervals. Unfortunately, we obtained for none of the coelomic fluid from each earthworm species change in titer of the treated virus on virus control (no virucidal activity for any of the time intervals).

provement of cell viability of infected and treated cells in comparison with virus controls was measured).

Antibacterial function of coelomic fluid

The observations with coelomic fluid of earthworm species: *Aporrectodea rosea*, *Lumbricus terrestris* and *Octolasion lacteum* showed that they do not have cytolytic or antibacterial activity. Only the coelomic fluid of *Eisenia fetida* affects microbial growth. Three bacteria strains were involved in the experiment. The inhibitory effect against *Aeromonas hydrophila* was the largest, with a 17 mm average diameter. Similar results were obtained for bacteria strains from genus *Pseudomonas*. *Pseudomonas luteola* has inhibition zone of 11.66 mm and *Pseudomonas fluorescens* with 11 mm (Fig. 2). The *Eisenia fetida* coelomic fluid has a bacteriostatic effect on the studied pathogenic microorganisms.

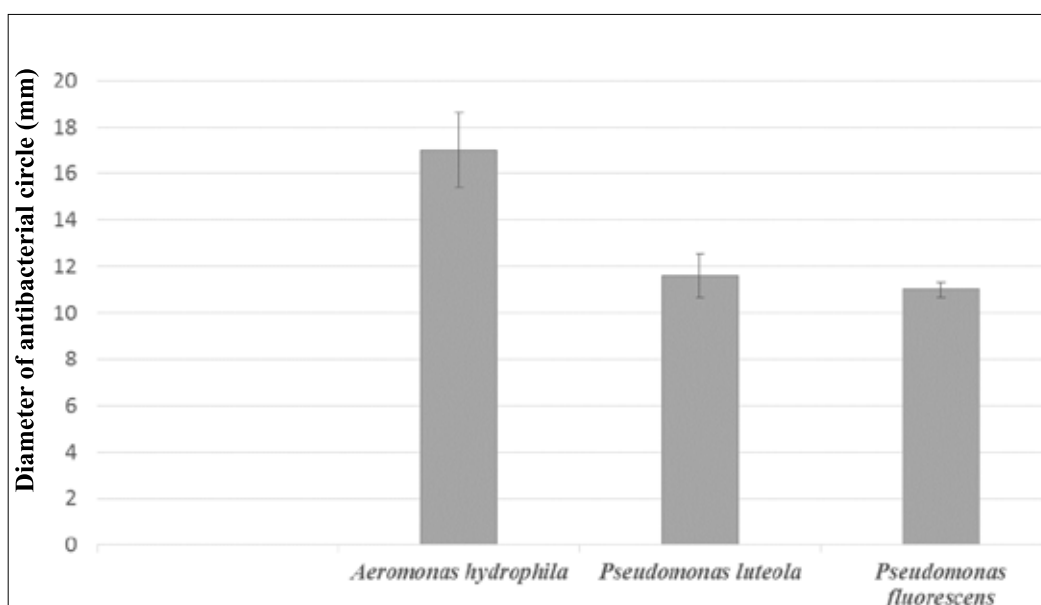


Fig. 2. Antibacterial activity of coelomic fluid of *Eisenia fetida*

Cell protection activity

The antiviral potential of the coelomic fluids against the replication of HSV-2 strain BA was tested. The obtained coelomic fluid from three of the earthworm species *Aporrectodea rosea* (Savigny, 1826), *Lumbricus terrestris* (Linnaeus, 1758) and *Octolasion lacteum* (Örley, 1881) was applied in concentrations ranging from 25 % (corresponding to their MNC) to 0.39 %. The obtained materials from the forth of the earthworm species *Eisenia fetida* (Savigny, 1826) was applied in concentrations ranging from 1.56 % (corresponding to its MNC) to 0.097 %. Unfortunately, no antiviral activity was found according to the modified MTT assay (no im-

Discussion

Few studies have revealed antibacterial activity of earthworm extract to *Pseudomonas aeruginosa* and *Pseudomonas pyocyanea* (Liu *et al.*, 2004; Wang *et al.*, 2007). To our knowledge, the current paper is the first exploration of the effect of earthworm extract against Gram-negative bacteria *Pseudomonas fluorescens*. The coelomic fluid of the earthworm *Eisenia fetida* (*Oligochaeta*, *Lumbricidae*) was demonstrated to possess antimicrobial activity directed against earthworm pathogenic bacteria *Aeromonas hydrophila* (Lassegues *et al.*, 1989; Valembois *et al.*, 1982). Antibacterial effect of *Dendrobaena veneta* (*Oligochaeta*, *Lumbrici-*

dae) on *Pseudomonas luteola* was observed by Arslan-Aydoğdu and Çotuk (2008). Until now, only few research papers regarding the antiviral activity of coelomic fluid from earthworm have been published. Lui *et al.* (2008; 2012) demonstrated antiviral effect against influenza and adeno viruses.

Our research revealed that only the coelomic fluid secreted from the dorsal pores of epigeic earthworm *Eisenia fetida* has bactericidal activities. The results indicated that the studied materials can affect the growth of Gram-negative bacteria from genus *Aeromonas* and *Pseudomonas* and the effect is bacteriostatic.

The antiviral experiments involved investigation of the cellular toxicity of the coelomic fluid of four earthworm species against the cell line used. Only the studied material from *Eisenia fetida* affected cell growth. Microscopic observation of the cell monolayer one hour after adding the dilutions of the coelomic fluid revealed changes in the monolayer integrity and cell morphology. This means that toxicity develops quickly and is probably connected with the disturbance of the cell membrane integrity and functions. Presumably, this quickly developing toxicity is due to proteins capable of forming pores into the membrane, such as lysenin (Sekizawa *et al.*, 1996) and eiseniapore (Lange *et al.*, 1997). Unfortunately, no activity against the replication of Herpes simplex virus type 2 (genital herpes) or its extracellular forms (virucidal activity) was observed.

Unlike vertebrates, earthworms lack real anti-bodies and efficient innate immune systems to defend themselves against invading foreign materials (Wang *et al.*, 2007). So, the coelomic fluid of *Eisenia fetida* plays a very important role in antibacterial defense (Valembois *et al.*, 1982). Coelomic fluid has various metabolic complements, which inhibit the growth of potent worm pathogenic bacteria (Lassegues *et al.*, 1989).

Further studies are required to estimate the mechanism of action of coelomic fluid on bacteria, viruses and eucariotic cells. Proper utilization of active peptide components of coelomic fluid may lead to discovery of a new way to manage bacterial or tumor cells growth.

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Inhibiting Effect of Growth Regulators on Plant Viruses

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Abstract

The effect of the growth regulator β -monomethyl ester of itaconic acid (MEIA) on two economically important viruses, namely tomato spotted wilt virus (TSWV) and tomato mosaic virus (ToMV), was studied in tomato cultivars. Tomato plants of Keti and VK1 lines were infected separately with each virus: Keti with TSWV and ToMV, and VK1 with TSWV, respectively. The tomato plants were treated with MEIA in a concentration of 5×10^{-4} M after inoculation with TSWV in one variant, and with ToMV in another.

An inhibiting effect was established on both viruses – TSWV and ToMV, when the tomato plants were infected and after that sprayed with MEIA solution. These initial results are a good reason to continue with the studies both for the establishment of the effect of growth regulators (MEIA in particular) on virus pathogens and the physiological indexes that prove the decrease in stress and injuries on tomato plants with virus diseases after treatment with MEIA.

Key words: growth regulator, inhibiting effect, TSWV, ToMV.

Резюме

Изследван е ефектът на растежен регулатор - β -монометил естер на итаконовата киселина (МЕИК), върху два икономически важни за доматиите вируси: на доматиената бронзовост (tomato spotted wilt virus-TSWV) и на доматиената мозайка (tomato mosaic virus - ToMV). Доматени растения от линиите Кети и VK1 бяха инфектирани поотделно с всеки от вирусите съответно Кети с TSWV и ToMV а VK 1 с TSWV. След инфектиране в един вариант с TSWV и в друг вариант с ToMV, доматиените растения бяха третирани с МЕИК в концентрация 5×10^{-4} М.

Инхибиращ ефект бе установен и върху двата вируса – TSWV и ToMV при инфектиране на доматиени растения и след това пръскане с разтвор на МЕИК. Тези първоначални резултати дават основание да продължат изследванията не само за установяване ефекта на растежни регулатори (в частност на МЕИК) върху вирусни патогени, но и за установяване на физиологични параметри, доказващи намаляване на стреса и повредите при вирусно болни доматиени растения след третирането им с МЕИК.

Introduction

Tomato spotted wilt virus (TSWV), *Bunyaviridae* family, *Tospovirus* genus and tomato mosaic virus (ToMV), *Virgaviridae* family, *Tobamovirus* genus cause yield reduction and deteriorate the quality of field and greenhouse-grown tomatoes. (Dikova, 2014a). The inhibition (full or partial suppression of virus multiplication, transport and activity in the host plants) with chemical resources acting, simultaneously as growth regulators is a perspective approach to the control of economically important viral diseases, such as tomato spotted

wilt and tomato mosaic. This is especially important for greenhouse production, where the smaller area will make the treatments economically profitable vs. field tomato growing. The inhibitors are antiviral substances that suppress or fully impede the replication of the viruses and the external manifestation of the symptoms. These substances include growth regulators and substances originating from different organisms (antibiotics, enzymes, antisera) or higher plants (Kovachevsky *et al.*, 1995). There are data on the inhibiting effect of fennel and lavender essential oils on plant virus when used at a

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dilution of 3000 ppm (Shukla *et al.*, 1989; Dikova, 2014b). Extracts of different plants, such as *Plectranthus tenuiflorus*, had antiphytoviral activity to TSWV, which can be attacked *in vitro*. When this extract was mixed with the virus inoculum for three hours, it inhibited the local lesion development in the indicator plant *Chenopodium amaranticolor* by 100 % (Othman *et al.*, 2004).

Phytohormones have been used to control some viral and phytoplasma diseases. For example, the treatment with kinetin before or right after inoculation with some plant viruses decreased viral multiplication and the intensity of symptoms in local infection and delayed plant perishing in systemic infection. For the time being, only gibberellin acid is used for the restriction of the *Sour cherry yellows virus* infection on cherry in field conditions (Stancheva, 2004).

MEIA is a growth regulator with a wide spectrum of activity, which increases the yield and improves the quality of various economically important crops. This compound with low toxicity is a derivative of naturally occurring plant metabolites. It is known that applied on cereals and leguminous, MEIA leads to a decrease in stress and injuries caused by abiotic and biotic stress factors. MEIA reduces the negative effect of some herbicides (Karanov *et al.*, 2001), induces systemic acquired resistance (SAR) in tomato plants (*Lycopersicon esculentum* L.) against a tomato mosaic virus (ToMV) (Krezhova *et al.*, 2010).

The objective of the study was to establish the effect of the growth regulator MEIA on tomato plants infected with two pathogenic plant viruses typical of tomatoes.

Material and Methods

The investigations were carried out on two tomato genotypes characterized by different expression of anthocyanins in plant vegetative parts. VK1 is a non- anthocyanin line, while Ketī has high anthocyanin content in all plant parts (Fig. 1).

We used the growth regulator β -monomethyl ester of itaconic acid (MEIA) in the trial for TSWV and ToMV inhibition. The stimulating effect of MEIA, which is a derivative of carboxylic acids, was proved on healthy tomato plants parallel to the trials for the establishment of the inhibiting effect on viruses. MEIA was applied after infecting the plants with TSWV in one variant, and with ToMV in the other. The plants were treated at the 3rd to 5th real leaf stage with MEIA solution in a concentration of $5 \cdot 10^{-4}$ M with an addition of Tween 80 0.05

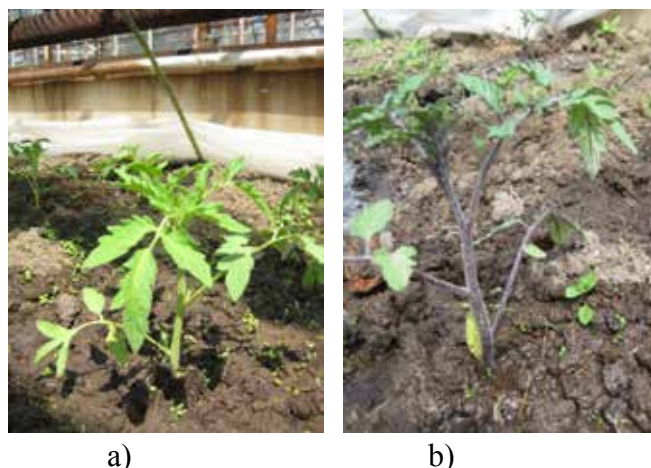


Fig. 1. a) line VK 1 ; b) line Ketī

% v/v tap water solution. Control plants were treated with Tween 80 0.05 % v/v tap water solution only.

TSWV infection was done by artificial mechanic inoculation with infectious material of pepper isolate of the same virus, replicated in tobacco plants – *Nicotiana tabacum* cv. Samsun NN. The trial was carried out with tomato seedlings, naturally infected with ToMV, probably by transmission of the virus with seeds of the Ketī line. We used virus-carrier seedlings without mechanical inoculation with ToMV and after that treated with MEIA the tomato plants which manifested symptoms. The tests for establishing the inhibiting effect of MEIA on TSWV and ToMV were carried out by the serological method DAS-ELISA according to Clark and Adams (1977). Tomato plants of lines VK1 and Ketī were analyzed in the following variants: 1. Healthy plants (negative controls for TSWV and ToMV); 2. Plants infected with TSWV and ToMV (positive controls for TSWV and ToMV); 3. Plants treated with MEIA; 4. Plants infected with TSWV or ToMV and subsequent treatment with MEIA. All trials for artificial inoculation of the tomato plants and the positive controls for both viruses were carried out according to Noordam (1973).

Results

The damages caused by TSWV and ToMV affected the shelf appeal of the fruits picked from the infected plants (Fig. 2a and 2b).

The growth regulator MEIA had a stimulating effect when applied on healthy non-infected tomato plants (Fig. 3, right). The leaves of the treated plants, compared to healthy non-infected and not treated with MEIA controls were larger and of darker green color (Fig. 3).



Fig. 2. Damages, caused by plant viruses on tomato fruits
a) - chlorotic concentric spots, caused by TSWV
b) - necrotic figures, caused by mixed TSWV and ToMV infection



Fig. 3. Effect of the growth regulator MEIA on tomato line VK1

Left - leaf of a tomato plant not infected with TSWV or ToMV and not treated with MEIA

Right - leaf of a tomato plant not infected with TSWV or ToMV but treated with MEIA

We established the inhibiting effect of MEIA on TSWV (Fig. 4 and 5) and ToMV (Fig. 6) by comparing the optical density values (the extinction values of samples from all tested tomato plants).

The inhibiting effect on TSWV was manifested in VK1 and Keti line, and on ToMV – in Keti, when the infected plants were treated with MEIA in a concentration of $5 \times 10^{-4} \text{M}$. The average extinction values of TSWV and ToMV infected tomato plants treated later with MEIA in a concentration of $5 \times 10^{-4} \text{M}$ did not exceed the values of the healthy plants more than 2.5 times (threshold value), therefore, they were not virus carriers. This phenomenon could be due to the effect of the growth regulators (Figures 4, 5 and 6). The values of the virus infected plants were manyfold higher than those infected and afterwards treated with MEIA. The viral concentration of TSWV and ToMV during the vegeta-

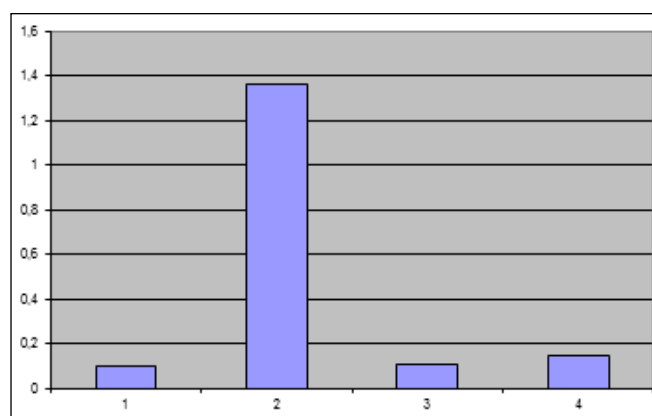


Fig. 4. Effect of TSWV infection and MEIA treatment on VK1 tomato line

On ordinate – Optical density (average extinction values for TSWV of tomato plants of the separate variants)

On abscissa – variants: 1. Healthy plants; 2. TSWV infected plants; 3. Plants treated with MEIA; 4. TSWV infected + MEIA treated plants

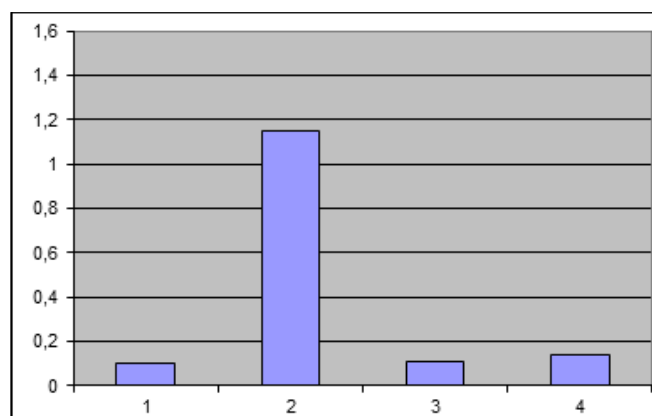


Fig. 5. Effect of TSWV infection and MEIA treatment on tomatoes cv. Keti

On ordinate – Optical density (average extinction values of TSWV infected tomato plants for the separate variants)

On abscissa – Variants: 1. Healthy plants; 2. TSWV infected plants; 3. Plants treated with MEIA; 4. TSWV infected + MEIA treated plants

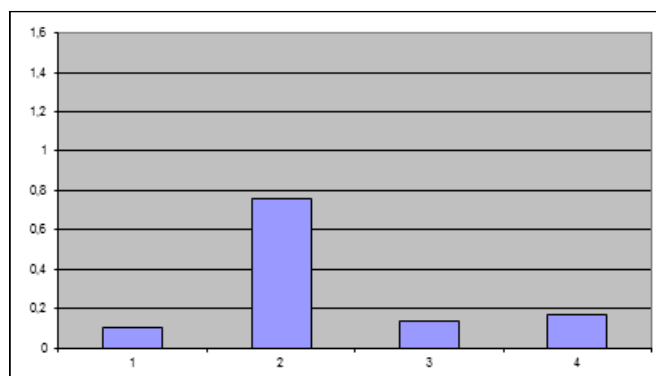


Fig. 6. Effect of ToMV infection and MEIA treatment on tomato line Keti

On ordinate – Optical density (average extinction values of ToMV infected tomato plants for the separate variants)

On abscissa – Variants: 1. Healthy plants; 2. ToMV infected test plants from the species *Nicotiana tabacum* cv. Samsun; 3. ToMV infected + MEIA treated plants from 03.09.2015; 4. ToMV infected + MEIA treated plants from 17.09.2015

tive period could be reduced with MEIA treatment. The results were encouraging to continue the investigations in view of decreasing the damages caused by TSWV and ToMV on the vegetative parts of tomato plants and their fruit, and guaranteeing a higher quality produce.

Discussion

It was found that anthocyanins had a protective effect with regard to abiotic and biotic stress (Krezhova *et al.*, 2010). There were no statistically significant differences in terms of virus inhibition both in the anthocyanin line and the one without anthocyanin. It is known that MEIA, applied together with herbicides with a different mechanism of action, can decrease the herbicide rate by half and still maintain its effect. Thus MEIA has a protective effect on mono- and dicotyledonous cultivated plants (Karanov *et al.*, 2010). The initial results gave a good reason to continue with the studies in order to establish the physiological parameters that prove the reduction of stress in virus infected tomato plants, following the treatment with MEIA.

Conclusion

β -Monomethyl ester of itaconic acid (MEIA) used as a growth regulator had an inhibiting effect on two most common viral pathogens on tomato: tomato spotted wilt virus (TSWV) and tomato mosaic virus (ToMV). It is necessary to conduct a future detailed study on the mechanisms of the protection effect. The use of MEIA growth regulator as a plant virus inhibiting tool accounts for efficient control aiming at regulating biotic stress in plants. Combined with the reduction of the harmful effect of herbicides, it may become very important for the overall protection of plants and the environment.

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WORDS ON THE LIFE AND WORK OF PROF. DR. PETER ANDONOV

Prof. Dr. Petar Andonov Stoynev was born in 1919 in the hamlet of Gorno Uyno, Kyustendil region. His remarkable life is a vivid example of how a poor peasant boy attained the highest levels of achievement in public health and science. Hopefully, his story will become an incentive for young people who, through hard work, perseverance and dedication, can achieve high goals.

Prof. Andonov's merits for the development of Bulgarian virology are indisputable. In 1957, having successfully defended a thesis in Moscow and ignoring the tempting offers for a career, he returned to Bulgaria. In 1958, the Ministry of Health entrusted him with organizing a separate Department of Virology at the Scientific Institute of Epidemiology and Microbiology (NIEM). Initially, the Department comprised the existing influenza, enteroviruses, smallpox and rabies laboratories, and the unit of electron microscopy, located in the main building. Shortly, new collaborators were recruited for the new units for hepatitis, cell cultures, echo and coxsackie viruses. Soon afterward, in 1960, the

team was reinforced with the arrival of new members, and more laboratories were built and equipped for respiratory viruses, biochemistry and physiology of viruses, measles and mumps. The facilities of the former Anti-Plague Institute were assigned for the needs of the Department of Virology. At the time, the existing premises were only few - former stables adapted into several primitive laboratories by the collaborators themselves. Every week the team would meet to discuss the work in each of the laboratories and share scientific news. In warm weather, discussions were held in the courtyard under a blooming chestnut-tree, or in a wooden shack in the cooler seasons.

As early as 1956, Prof. Andonov managed to convince the leadership of the Ministry of Health of the need of a new building. His dream was materialized within a short term with the construction of the Department of Virology at 44-a Stoletov Boulevard. Later, after the onset of the AIDS problem, another special building was erected in the yard of the institute.

Owing to the efforts of Prof. Andonov, a network for epidemiological prophylaxis and prevention was created in Bulgaria. On his initiative, sanitary-epidemiological stations were set up in the major cities, which were specialized units for virological investigations.

The elected Specialized Council of Virology at the Higher Attestation Commission under the chairmanship of Prof. Andonov had an important role. In 1962, Prof. Andonov was invited to organize a course in virology at the Faculty of Biology, Sofia State University, and was elected professor of virology. The faculty became the source of young new specialists. Thus, Prof. Andonov successfully secured sources of both virology specialists and scientific potential, which took Bulgarian virology to a leading position in Eastern Europe.

In view of his outstanding qualities, the state assigned Prof. Andonov with a number of important positions: Deputy Chairman of the Medical Academy, Deputy Minister, Rector of the Medical University, Editor-In-Chief of *Zdraven Front* newspaper, and other responsible posts. Deservedly, for his successful work he was awarded with the highest state and academic titles.

Prof. Andonov's prolific and productive organizational experience, however, does not come short of his qualities of a scholar, which won him international prestige. He gained recognition by becoming member of a number of committees of the WHO, including the International Committee on Taxonomy of Viruses. Prof. Andonov has taken part in many international events and editorial boards of leading academic journals. His active research work was published in more than 200 articles and reviews. He is the author of the first Textbook of General Virology. Prof. Andonov is author, co-author and consultant to many major achievements of other members of the Department of Virology, and of more than 15 patents and inventions. The main directions of his extensive research focused on the prophylaxis, diagnosis and treatment of viral pathologies in Bulgaria. New methods were implemented fairly quickly in the Department. In most cases, teams worked hard under his leadership on the development of vaccines and therapeutic agents.

Based on original methodology, the team Andonov, Dundarov and Bakalov started the preparation of antiherpes type 1 and 2 vaccines, which by far outperform all other antiherpes vaccines. They

became the only commercialized vaccines in the world, used widely with great interest abroad. Owing to them and the efficient drug PANDAVIR, the lives of many new-born babies was saved, as well as the sight of hundreds of patients and thousands of patients with severe forms of genital, labial and skin herpes.

It is worth mentioning that the vaccines, diagnostic and therapeutic agents produced over the years in the Department of Virology contributed to the low morbidity and mortality rates. In that respect, Bulgaria was held up as a positive example to most countries in the world.

This enormous and fruitful work was covered in thousands of national and foreign journals and was popularized at local and international events.

The year 2018 will mark the 70th anniversary of the Department of Virology at the National Centre of Infectious and Parasitic Diseases, an event which we hope will receive well-deserved recognition and the role of Prof. Andonov will be highly appreciated.

Prof. Stefan Dundarov, MD, DSc

Prof. Hristo Odysseev, MD, DSc

Fourth National Congress of Virology with International Participation Days of Virology in Bulgaria

The Fourth National Congress of Virology with international participation, also known as Days of Virology in Bulgaria, was held from 18 to 20 May 2016 in the Great Hall of the Bulgarian Academy of Sciences. The event was organized by the Bulgarian Society for Microbiology at the Union of Scientists in Bulgaria. This major scientific forum was chaired by Academician Angel Galabov, world-renowned virologist. A total of 50 research papers were presented, devoted to the latest advances in the field of medical, veterinary and plant virology. Eminent scholars such as Prof. Francis Delpeyroux from France, Prof. István Tóbiás from Hungary, Prof. Anna Papa from Greece, Prof. Rajarshi Gaur from India attended the congress. Bulgarian virologists were represented by the teams of Academician Angel Galabov and Prof. Tatiana Cherveniakova, Prof. Radka Argirova, Assoc. Prof. Anthony Stoev, Assoc. Prof. Bistra Dikova, Prof. Raiko Peshev, and many others. The scientific forum ended with a round table discussion on "Training in virology at universities".

The Fourth National Congress of Virology with international participation, also known as Days of Virology in Bulgaria, was held from 18 to 20 May 2016 in the Great Hall of the Bulgarian Academy of Sciences. The event was organized by the Bulgarian Society for Microbiology at the Union of Scientists in Bulgaria. Chairman of the major scientific forum was the world-renowned virologist Academician Angel Galabov.

In his opening speech, Academician Galabov emphasized that *"We are happy to be able to get together and discuss our pursuits and achievements in the field of virology in the period after the Third Congress in 2012. We are now going to hear about recent scientific advancement in Europe, Asia, the Balkan region, and Bulgaria, of course, as well discuss current issues relating to the teaching of virology."* Academician Galabov presented the journal *Acta Microbiologica Bulgarica*, issued by the Bulgarian Microbiology Society. The journal covers the latest advances in all branches of microbiology, and its current issue is dedicated to the Fourth Congress of Virology.

At the opening of this important scientific event, Hristo Naidenski, a corresponding member and director of the Stephan Angeloff Institute of Microbiology at the Bulgarian Academy of Sciences, also addressed the congress participants by focusing their attention to the main themes of the plenary papers, scientific communications and posters, which featured the latest problems of general, med-

ical, veterinary and plant virology. Mr. Naydenski wished the participants of the Congress productive and beneficial work, both for science and society.

Successful days for virology in Bulgaria were wished by Prof. Paskal Zhelyazkov, director of the National Diagnostic and Research Veterinary Institute "Prof. Dr. Georgi Pavlov" in Sofia. Prof. Zhelyazkov said that despite the financial constraints on Bulgarian science, the specialists at the institute headed by him strive to maintain the highest levels of competence in their work and be among the first in the combat against infectious diseases, trying to work to the benefit of society.

The prelude to the forum presentations ended with an exciting speech given by Academician Angel Galabov, dedicated to the work of 97-year-old Professor Petar Andonov, whose merits for the development of medical virology and training in virology in the Faculty of Biology of Sofia University are significant and worthy of deep respect.

In the remote 1958, Prof. Andonov organized a separate Department of Virology at the present-day National Centre of Infectious and Parasitic Diseases. On his initiative, hygienic epidemic stations (HES) were founded in the major cities, with specialized units for virological investigations. It was him who set up and chaired the Specialized Scientific Council of Virology at the Higher Attestation Commission. In 1962, Prof. Andonov introduced a course in virology in the Department of Biology, Sofia University, making this research unit a hotbed

for young virologists. Over the years he held many responsible positions: Deputy Minister of Public Health, Deputy Chairman of the Medical Academy, Rector of the Medical University, editor-in-chief of the journal "Health Front". For his scientific contributions Prof. Andonov gained international acclaim, sat on many committees of the World Health Organization, including the International Commission on Taxonomy of Viruses. He authored the first Textbook on General Virology, as well as more than 200 scientific articles and reviews. He co-authored over 15 scientific inventions. Today, despite his venerable age, Prof. Petar Andonov continues to be vividly interested in innovations in his area of speciality, including the present scientific meeting of eminent virologists from Europe and Asia.

The first plenary lecture at the congress was delivered by Assoc. Prof. Nikolai Petrov, virologist from the Institute of Soil Science, Agrotechnologies and Plant Protection "Nikola Poushkarov" at the Agricultural Academy and the Institute of Microbiology "Stephan Angeloff" at the Bulgarian Academy of Sciences. Assoc. Prof. Petrov presented the role of the so-called small interfering RNA in the fight against human enteroviral infections. He shed light on the defence mechanism of gene silencing - a process in which gene expression is blocked. Each organism is composed of genes (heredity carriers) which are programmed so that certain features become expressed at a certain time of development of the organism. This process occurs gradually, with some genes being blocked, while others are not. When it is time for a gene to be expressed, gene silencing does not kick in. Scientists seek to use this normally functioning mechanism in organisms for a variety of purposes. One of them is to block genes of pathogenic viruses (causing diseases). Thus, the virus cannot develop and is prevented from invading the body. When tumours develop, the virus could again be blocked by means of gene silencing, thus suppressing its expression. In drug tests, it is possible to block certain genes, one at a time, and thus determine at what stage of development, on which gene, a specific drug will act. This is the path of finding new applications for different drugs. Currently, researchers are close to solving the problem of blocking the human immunodeficiency virus (HIV) by the mechanism of gene silencing.

One of the papers relating to this mechanism was delivered by Prof. Rajarshi Gaur from India, who presented the results of his team's studies on begomoviruses responsible for the reduced yields

of a number of vegetables in the world, leading to significant economic losses. The data quoted by Prof. Gaur show that begomoviruses are expanding their host range. Therefore, researchers seek to find an efficient way of resistance to the viral invasion. This is the aim of the gene silencing strategy developed in India, which may be used for control of begomoviral symptoms in plants. *"This problem is very important not only for India but also for Europe. We are working on a joint project with Dr. Nikolai Petrov and are preparing a book authored by specialists from around the world. In Bulgaria the book will come out in July this year - underlined Prof. Gaur. - It is a book about the origins and history of plant viruses, about which crops become infected and how farmers can fight plant diseases, ensuring their protection by means of various technologies"*.

Special interest provoked the paper of Prof. István Tóbiás from Hungary, which reviewed the periodic appearance of the virus causing tomato spotted wilt (TSWV). The fight against that virus initially involved insecticides and insect traps against thrips carrying the infection. Later on, it also included attacks against weeds that are hosts for the virus. Then, a resistance gene to this particular virus was introduced in some varieties of pepper. However, sporadically in 2010, then more frequently in 2012, the resistance seemed to be overcome by a strain of the same virus. *"In Hungary, tomato and pepper production is extremely important for the country, said Prof. Tóbiás. That is why we are looking for ways to deal with the virus. The aim of our research work is to find new sources of genetic resistance in tomatoes and peppers"*.

The question of plant resistance against aggressive phytopathogenic viruses was examined from various angles in a series of scientific communications by renowned specialists. Assoc. Prof. Bistra Dikova of the Institute of Soil Science, Agrotechnologies and Plant Protection presented the spread of cucumber mosaic virus and the diseases it causes in aromatic medicinal plants with have promising perspectives for essential oil extraction. Her main recommendation was that special attention should be paid to the damage to plants caused by the cucumber mosaic virus. Assoc. Prof. Antoni Stoev from the same institute discussed some peculiarities of viral plant diseases that are of importance to agro-ecosystems. In his view, the extent of damage in plants depends both on the aggressiveness of the viruses and on the possible stress on the environment. *"We cannot destroy viruses but*

we must learn to coexist with them”, emphasized Assoc. Prof. Stoev, quoting the founder of plant virology in Bulgaria, Prof. Dr. Dimitar Atanasov.

Another large group of papers dealt with viral aggression in animals, leading to diseases that have a significant economic importance, as well as diseases dangerous to humans: infectious bovine rhinotracheitis; mucosal disease; chlamydia; rickettsia, etc. Prof. Raiko Peshev from the National Veterinary Institute reviewed the features of malignant catarrhal fever, studied by his team in Sofia and Varna zoos two years ago. He also pointed to the possible measures to curb the disease in zoos and farms, as well as to block the routes leading to infections in humans.

The question of the eternal fight between micro- and macro-organisms - between viruses and Nature’s most biologically complex organism, *Homo sapiens* - was raised at this congress again. The participants showed a special interest for the paper of Prof. Francis Delpeyroux of the Pasteur Institute in Paris. Prof. Delpeyroux, who is a leading specialist in genetics of enteroviruses, discussed the current possibilities of control and eradication of polioviruses of the enterovirus species group, causing poliomyelitis (polio). While working with his team on the island of Madagascar, in Nigeria and Cameroon, he studied the effect of the combination of coxsackieviruses with Sabin’s polio vaccines. *“We need to make more effective and safer vaccine that will save more lives, said Prof. Delpeyroux, because we know how to manipulate the genome.”* He emphasized that *“I am really delighted to work with Academician Galabov, who is a leading European specialist in discovering drugs against enteroviruses. Studying the effects against viruses together is a pleasure for me. We both believe that it is important to find effective drugs to help people”*. One of the future intentions of the eminent French professor is to recruit young people in his team, who are interested in science work on the creation of new antiviral therapies. He is convinced that researchers in the field of virology should direct their efforts in favour of different countries and share their experience - scientific, life, human. *“This is important if we want to make the world a better place”*, said Prof. Delpeyroux. In the same vein is his vision on the possibility of introducing new criteria for the preparation of vaccines: *“This is both difficult and easy. It is important to have the support of a large international organization of public health and be careful what you are doing. Each molecule must undergo a test before being used on patients”*. For

virologists this is a guiding ethical rule, as was repeatedly affirmed during the congress.

A communication presented by a team of young researchers headed by Academician Angel Galabov caught the attention of virologists with studies on the therapeutic options for enterovirus-like infections. Enteroviruses are easily changeable and can become resistant to every drug used against them, which is observed in the use of monotherapy courses. Combinations of two agents may also result in virus resistance (so-called double resistance). The new method is a consecutive alternative application of combinations of anti-enteroviral substances with known mechanisms of action. Researchers have witnessed an optimal antiviral effect with a triple combination, which is of tremendous practical importance for the management of diseases such as summer flu, meningitis, eye infections, pericarditis, myocarditis, etc.

A serious factor against the invasion of HIV infection has been the introduction of the so-called protease inhibitors therapy, reported by Prof. Radka Argirova. According to her, these products have changed the entire HIV/AIDS epidemic, and have transformed it from an inevitably fatal disease into a chronic manageable condition. Since 2012, there have been talks about the body’s ability to fight off HIV infection through different routes of eradication. Research in this direction also benefits the battle against hepatitis C infection, is Prof. Argirova’s strong belief.

The communication of Prof. Anna Papa from the Department of Microbiology at the Medical University of Thessaloniki focused on infections caused by arboviruses in the Mediterranean countries in recent years. Among the main factors leading to the spread of these diseases Prof. Papa pointed the change in the environment and globalization. Dealing with the problem, in her opinion, must involve competence and greater awareness of the health care providers, correct reporting of unusual cases and cooperation between experts from different countries around the world.

Very useful for clinical practice was the study on viral gastroenteritis, carried out by a team of Prof. Tatiana Cherveniakova from the Specialized Hospital for Infectious and Parasitic Diseases “Prof. Ivan Kirov” in Sofia. The study group included 22 students (17 boys and 5 girls) from the Professional High School of Computing and Technology Systems in Pravets, aged 14 to 18, with severe gastroenteritis. The therapeutic approaches successfully implemented were: oral and parenteral rehydration,

medication for the symptoms of gastroenteritis, and appropriate diet.

An analysis of data obtained from virological studies and from the clinical course of influenza and respiratory syncytial virus infections in patients at the Hospital of Infectious Diseases “Prof. Ivan Kirov”, Sofia, was presented by Ivan Ivanov, together with the work of a team of researchers from the same hospital, from the Medical University in Sofia and from the National Centre of Infectious and Parasitic Diseases. For the period from December 2015 to March 2016, the team determined A (H1N1) pmd09 and type B as the predominant influenza strain, which caused infections requiring hospitalization. For the same period, respiratory syncytial virus was the primary etiological agent among children under 5 years of age, leading to prolonged illness and slow recovery.

Interesting results of recent studies with a potential for new developments were presented by a group of virologists from Varna. The research work of Assoc. Prof. Lilia Ivanova, head of the Department of Microbiology and Virology at the Medical University, Varna, and of Zhivka Stoykova from the Clinical Laboratory at “St. Marina” Hospital showed that cytomegalovirus infection is most widespread among the population in North-Eastern Bulgaria and occurs mainly in the first years of life. Another study reported high incidence of Epstein-Barr virus infections also among the population of North-Eastern Bulgaria, with the greatest risk of developing Hodgkin’s lymphoma in the first three years following the infection.

A new challenge in virology is occult hepatitis B, studied by researchers from Varna among 55 patients at “St. Marina” Hospital. All patients

showed clinical evidence of liver dysfunction and elevated liver enzymes. A comparative analysis with previous studies allowed the authors to confirm the expected result that patients with HBV-infection and liver disease are the most vulnerable.

It is difficult to enumerate all the reports which discussed the most recent developments in the major branches of virology. The congress proceeded in a highly academic atmosphere and ended with a discussion on the question of the public benefit from the knowledge of virology and training in this discipline in the universities in Bulgaria. According to Assoc. Prof. Lilia Ivanova, different universities give priority to different aspects of microbiology, and this situation must change. *“Virology and bacteriology are studied on an equal basis, and all our lectures accentuate the need for preventive and therapeutic strategy against diseases,”* said Assoc. Prof. Ivanova. *Currently, there is a tendency where doctor virologists are replaced with biologists and molecular biologists. This means loss of the much-needed clinical opinion in therapy. A doctor-virologist is the one who can best interpret laboratory data, taking into account the clinical manifestations of the individual patient. It does not suffice to make a diagnosis. It takes at least two methods to verify the results to ensure their validity.”*

From beginning to end, this great scientific forum was filled with practical ideas of benefit for people, as well as with innovative, inventive spirit, and creative, bold outlook into the future. So, it will not be an exaggeration to say that the Fourth National Congress of Virology has, in a way, become a gateway to new scientific conquests.

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