

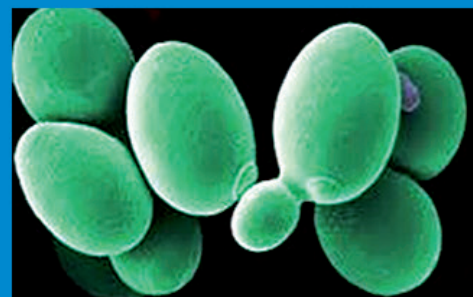
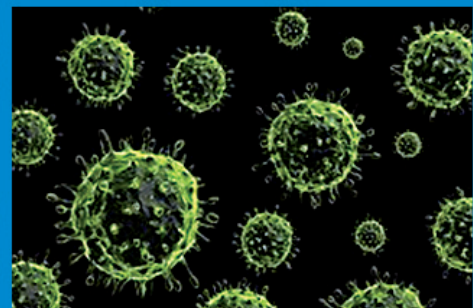


Volume 35, Issue 2, 2019

ISSN 0204-8809

ACTA MICROBIOLOGICA BULGARICA

Bulgarian Society for
Microbiology
Union of Scientists in
Bulgaria



Acta Microbiologica Bulgarica

The journal publishes editorials, original research works, research reports, reviews, short communications, letters to the editor, historical notes, etc. from all areas of microbiology

**An Official Publication
of the Bulgarian Society for Microbiology
(Union of Scientists in Bulgaria)**

Volume 35 / 2 (2019)

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Review

Peste des Petits Ruminants

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Abstract

Pest des petits ruminants (PPR) is a disease first described in 1941 in West Africa. It is caused by a virus belonging to the family *Paramyxoviridae*. The virus is highly virulent for goats and sheep. There is no virus carriage. The incubation period is 5-6 days. Mortality is high. Transmission of infection is through close contact between sick and healthy animals – infection occurs mainly through the respiratory tract. In pathogenesis, the lymphatic system plays a major role. The infection predominantly affects small ruminant flocks. Clinical symptoms are mainly lesions on the muzzle, nose and urogenital tract. Diagnosis is based on characteristic clinical and pathological anatomical evidence. Stringent measures are necessary to isolate and quarantine risky flocks and farms.

In connection with a PPR epizootic in Syria, the import of lambs from our country was terminated. The team of Bulgarian specialists sent to Syria proved that Bulgaria had nothing to do with the epizootic in that country. In 2018, the dangerous disease of sheep and goats was detected in Bulgaria. Data reported in the press and other media seriously differed from the well-known characteristics of the Pest des petits ruminants infection.

Keywords: Pest des petits ruminants (PPR), *Paramyxoviridae*, epizootology.

Резюме

Чумата по дребните преживни (Pest des petits ruminants) е заболяване установено за първи път през 1941 г. в Западна Африка. Причинителят е вирус от семейство *Paramyxoviridae*. Вирусът е силно вирулентен за кози и овце. Няма вирусоносителство. Инкубационният период е 5-6 дни. Смъртността е висока. Предаването на инфекцията е чрез тесни контакти между болни и здрави животни – зразяването е главно по респираторен път. В патогенезата главна роля изпълнява лимфната система. Епизоотологията е предимно стадна. Клиничната симптоматика са главно лезии по муцуната, ноздрите и уrogenиталния тракт. Диагнозата се поставя въз основа на характерните клинични и патологоанатомични данни. Предприемат се строги мерки за изолация и карантина на рисковите стада и ферми.

Във връзка с епизоотия от това заболяване в Сирия е прекратен вноса на агнета от нашата страна. Изпратенуият български екип от специалисти доказва, че нашата страна няма нищо общо с тази епизоотия. У нас през 2018 г. е обяено наличието на това опасно заболяване по овце и кози. От обявените в пресата и други медии данни се установяват седириозни различия от общоизвестните характеристики на чумата по дребните преживни.

Introduction

Notwithstanding existing difficulties in the scientific area, Bulgarian veterinary science has always sought to rise to the challenges posed by its profession and by the state regarding livestock wellbeing not only

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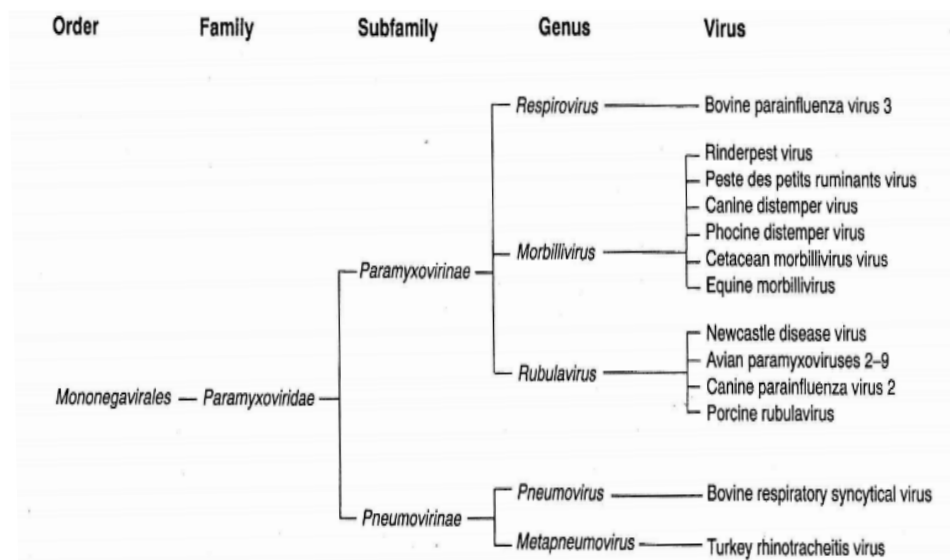


Fig. 1. Classification of parvomyxoviruses with emphasis on those of veterinary importance

in terms of trivial contagious diseases peculiar to a region but also against exotic diseases (Masalski, 1991; 2008).

It is only natural that along with the changes due to globalization exotic infectious diseases, once endemic, are now becoming a real global threat. Precisely for that reason, the knowledge about such diseases should be brought up to date so that adequate preventive and combat actions can be implemented. Undoubtedly, the requirements regarding the relatively novel disease worldwide, Peste des petits ruminants (PPR), are imperative. The disease was first reported in 1941 in Côte d'Ivoire, West Africa and is also known as Pseudorinderpest, Stomatitis-pneumoenteritis complex, Kata in Nigeria (Gargadannec and Lananne, 1942). It is endemic across the sub-Saharan countries, the Arabian Peninsula and some Middle East countries (Braide, 1981; Maztin, 1983; Taylor, 1984).

The cause of the disease is a virus belonging to the family *Paramyxoviridae* (Fig. 1) (Gibbs *et al.*, 1979). Although it shares characteristics with the bovine plague virus, the two viruses are not completely identical and are differentiated by cross-seroneutralisation (Geering, 1986). The epizootiology of the disease is characterized by certain peculiarities depending on the virus specificity, the host animal species - sheep and goats only, and on the nature of the disease process. The virus is highly virulent for sheep and especially high for goats and the young animals of those species. After recovery, there is no viral persistence in the body, therefore there is no carriage of the virus or intermediate carriers (vectors), and the role of the wild animals is insignificant as contact with domestic animals is only short and infrequent. The virus found in the excretions and secretions from infected animals does not survive longer than 1-2 days in the external

environment. The incubation period is short but the infected animals start shedding the virus several days after its onset in the absence of clinical signs. The clinical signs themselves are clearly expressed and the disease terminates within a short period of time with high mortality. All this determines the way the infection is transmitted and spread. Transmission occurs in extremely close contact between sick and healthy animals, and indirect transmission via feed, water or vehicles is almost impossible. A major source of contagion is infected animals introduced into a healthy flock. Epizootiological peculiarities make it predominantly a flock disease that does not tend to spread out of the affected flock or holding, although it spreads rapidly to almost all animals within the flock.

The cause of the mass disease in the flock is the way the virus is released, which initially occurs through nasal and conjunctival discharge and aerosols from the respiratory tract, and later, after the appearance of clinical signs, through faeces and urine. Although virus shedding and infection transmission is possible in all these routes, the primary mode of infection is through the respiratory system. Susceptible animals contract the disease by inhaling virus-containing droplets excreted by the sick animals in amounts sufficient to cause infection. This is the reason why it spreads at a great speed within the affected flock. In addition, contact between the sick and healthy animals is also facilitated by the high mobility of the sheep within the flock. In the countries where the disease is endemic, the spread within or across borders occurs through uncontrolled animal trade, and in particular through the formation of assembly flocks in preparation for their sale or by assembling animals in centres after their purchase from different sources and subsequent distribution to new owners. Such was the epizootic of PPR in Syria in 1987.

Pathogenesis. After inhalation of the virus, multiplication occurs in the pharyngeal and mandibular lymph nodes. Viraemia develops within 3 days and the virus spreads to other lymphoid organs, including the mucosa of the respiratory and digestive tracts. Necrotic processes occur in the lymphoid tissue and, as a result, animals develop leukopenia and immunosuppression. During the acute stage, the virus is shed in large quantities, but after the temperature returns to normal viral shedding also declines.

Clinical signs. The incubation period lasts from 5 to 6 days followed by sudden fever with a rise in temperature to 40-42°C and clinical signs are manifested 1 to 2 days thereafter. Fatigue, loss of appetite, serous nasal discharge, which becomes mucopurulent, are observed. The mucous membranes are red, breathing rate increases, constipation occurs. Three to four days after the onset of fever, lesions affecting the muzzle, nose, and urogenital tract begin to develop. Those in the oral cavity are accompanied by abundant salivation. Conjunctivitis and ocular discharge are one of the most common traits at this stage in the development of the disease. In the beginning, lesions are small necrotic areas. Within a short time, however, their number increases, they increase in size and merge. After removal of the necrotic tissue, uneven, well-defined erosions are found underneath. At this stage, the animals appear to be very sick. Their breath is putrid, the breathing is difficult with marked signs of tracheitis and pneumonia. The onset of diffuse diarrhea approximately coincides with the second day of the onset of mucosal changes. The faeces are dark, foul-smelling of rotting flesh, sometimes containing mucus, fragments of necrotic mucosa and blood. Severe leukopenia facilitates secondary pasteurella, streptococcus, salmonella and E. coli bacterial infection. Mortality is high, reaching 70-90% in young animals and goats and death occurs within 10 days of infection. The recovery of the few surviving animals is slow and takes a long time.

Pathological-anatomical changes. The carcasses of the dead animals are dehydrated, exhausted, soiled by loose stools and malodorous. Erosions of the conjunctiva, muzzle and nose can be observed (Fig. 2). In the oral cavity, erosions, ulcers and necrotic foci on the inside of the cheeks, tongue, gums, pharynx and the esophagus are found. The necrotic matter is gray, bran-coloured and easily removed, leaving an ulcerated reddish surface underneath (Fig. 3). Stomach mucosa is hyperemic, hemorrhagic and necrotic. Often, the



Fig. 2. Mucopurulent discharges from the eyes and nasal cavity



Fig. 3. Erosions and ulcerations on the tongue and soft palate

necrotic tissue has fallen off, and there are ulcerated areas underneath. Significant changes in the large intestine are manifested in severe hyperaemia, bleeding and necroses lending the folds a look of tiger stripes, likened by some to zebra stripes. Changes in the cecum at the sites of passage into the colon and into the rectum are also considerable. The lymph nodes are enlarged and hyperemic, the spleen is enlarged and with focal necrosis

Nasal mucosa is covered with a thick mucopurulent mass, and long strips of hyperemic mucosa can be seen in the trachea. The apical segments of the lungs reveal bronchopneumonic changes. The heart is covered with hemorrhages. Hemorrhages, necrosis and ulcers are also found in the mucous membranes of the urinary tract.

Diagnosis. Putative diagnosis is based on the characteristic clinical and pathological-anatomical features, and on the analysis of the epizootiological data. Specimens for life-time laboratory diagnosis confirmation are conjunctival and nasal discharge, rectal and buccal swabs. Anticoagulants are added to the blood samples. They are taken when the clinical

signs are most prominent. Suitable specimens for examination are those from the mesenteric and bronchial lymph nodes, spleen, colon and lung from dead animals. All samples must be fresh and stored at refrigeration temperature. The virus is isolated in tissue cultures of lamb kidney cells or VERO cells. The cytopathic effect occurs not earlier than the 5th day after inoculation. Virus confirmation is also possible by detecting it in specimens when encountered with standard positive sera through different reactions. Serological diagnosis reactions are based on the detection of antibodies in sick animals and in those surviving the viral infection. For that purpose, blood samples are taken during the illness and 2-3 weeks afterwards to determine the antibody dynamics. The presence of antibodies is confirmed in blood serum four years after the viral infection (Durojaiye and Taylor, 1984). Reversed arrangement of components is performed. The sera to be tested are associated with a known standard antigen derived from the PPR virus. Laboratory tests used for viral antigen detection are direct immuno-staining, agar-gel immunodiffusion, and ELISA among others. Virus-neutralization, indirect fluorescence assay, competitive ELISA and other tests are used for the detection of antibodies. RT-PCR is used to detect the presence of viral genome (OIE, 1992).

Prevention and control. PPR control measures depend on whether the disease is endemic or has newly emerged in a particular country. In countries where it is detected for the first time, radical measures are taken to eliminate all affected animals in the flock or holding, destroy their carcasses and afterwards carry out appropriate disinfection. Stringent measures are established for spatial isolation and quarantine of risky flocks and farms, which are placed under constant surveillance. In those countries where the disease is endemic, measures are implemented to limit uncontrolled movement of sheep and goats as well as mass vaccinations. The close antigenic kinship between the PPR virus and the bovine plague virus allows the use of a vaccine prepared from bovine plague vaccine strains inducing long-term immunity (Geering, 1986). Recent studies have yielded vaccines consisting of attenuated PPR virus strains inducing immunity for at least 18 months (OIE, 1992). It should be borne in mind that despite the success of immune prophylaxis, many of the affected countries are unable to eradicate the disease, which proves the disease to be difficult to fight and requiring a lot of effort and resources.

That is why the countries at risk must take effective measures through strict border control and mandatory meetings and consultations with the responsible veterinary services from neighboring countries with established virus outbreak in order to prevent cross-border transmission.

Our experience

It is well known that at the time of intensive sheep breeding in Bulgaria Syria was our main partner in exporting tens of thousands of lambs, suckling lambs and sheep. At the time of the first PPR outbreak in Syria (originally detected in assemblages of animals from different countries), it was alleged that the source of infection were the Bulgarian animals. Imports were suspended and due money transfers were blocked. The investigation conducted on Syrian territory proved the untenability of those accusations by highlighting several circumstances. The key argument was that the disease was circulating in the African countries in a west-to-east direction and had not reached the European continent, therefore Bulgaria, where it was not found either in individual farms or in assembly centres along the Black Sea coast in preparation of the animals for export. In addition, after inspection of documents, cattle centres and holdings, surveys and autopsies in Syria, it was established that the actual disease was peste des petits ruminants, confirmed by isolation of the virus at the reference laboratory in Brendbury, England, in samples taken and sent personally by FAO representative Dr. Singh, with whom we met and talked in person. Wrong actions and failings were also found in relation to the placement and distribution of imported animals. After unloading from the ships, animal were taken to assembly centres in the city of Hamas, East Syria, and then distributed to fattening farms, both in the city itself and in nearby and further distant areas of the country. In the holdings, imported animals were placed together with natively-bred animals as well as with animals imported from other countries, including Hungary. The same was true of small farms, where 20-30 young ovine animals were kept for further fattening. Young ovine animals arriving from Bulgaria and Hungary, where the disease did not exist, became infected on the territory of Syria, and, being more susceptible, suffered more severely than local sheep breeds, creating a deceptive impression that the disease had been imported with them. The farms where imported animals were fattened did not comply with the “full-empty” principle and animals from previous

lots were grouped together with animals from new lots. These and other arguments gave us reason to refute the allegations of the Syrian services against our country. Furthermore, we acknowledged that the strategy and tactics of the struggle for the eradication of the disease was within the competence of the national veterinary services and dependent on the epizootic situation, the structure of the sheep breeding, the economic and geographic features of the country and the rules generally accepted for fighting that dangerous disease. We competently proved that our country was unaffected by the peste des petits ruminants disease and facilitated the agreement for payment of the blocked \$ 3 million funds, which the Damascus Central Bank instructed the UBAF in London to transfer to the Bulgarian side. The restricted import of sheep, suckling lambs and lambs from Bulgaria was also restored. At the request of the Syrian veterinary authorities, we presented them in writing and in detail with these findings and relevant recommendations for the protection of animals imported from Bulgaria against this disease. Later communication from the Ministers of Agriculture and Trade revealed that the infection had been imported through smuggled livestock from Lebanon.

This case confirms that the epizootic characteristics of the disease require a serious approach to diagnosis and outbreak announcement, especially when the suspected occurrence is in a country previously unaffected by the disease.

In our country, which was unaffected by PPR, this dangerous disease was detected in sheep and goats in several villages in 2018. However, there are no research reports from the scientific community, despite the importance of the disease and the economic losses incurred. This is also necessary as to identifying the source of the infection, the route of the infection into the flock, the characteristics of epizootiology and the causative agent, as well as the measures for its eradication. The reports in the daily press and media appear to differ seriously from the well-known characteristics of PPR discussed herein. Our experience is related to the disease that originated in Syria. This gives us reason to

announce some important details, all the more so that our country was accused of being the source of the infection. Investigations and their findings may be in favor of serious scientific research by the veterinary scientific community. Their results should offer proper assessment of the situation that occurred in order to avoid similar complicated state of affairs in the future. The nation needs a precise and competent response from the veterinary science. Lack of such will always leave the public and professionals in doubt as to the origins of PPR occurring for the first time in a European country without elucidating the source of infection. All the more so, given the drastic and extreme measures taken to limit the disease administratively thus announced.

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Antiviral Activity of Dipyridamole in Experimental Viral Infections in Mice

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Abstract

Single application of the interferon inducer dipyridamole administered intraperitoneally or *per os* demonstrated a marked antiviral effect in mice with experimentally induced alphavirus infection – Semliki Forest (SFV) virus-caused encephalitis, applied orally in influenza virus A/Puerto Rico/8/34 (H1N1) and B/Lee/40 – respiratory infections, and herpes simplex virus type 1, strains Lennett and Leningrad 2 – encephalitis, and HSV-1 strain Leningrad 2-caused skin infection. In brain and respiratory infections the antiviral activity was manifested *via* the protection index, a decrease in the cumulative mortality percentage, and by the prolongation of the mean survival time. In the HSV-1 dermatitis suppression of symptomatology was observed.

Keywords: dipyridamole, interferon inducer, mice, SFV, HSV-1, influenza viruses A and B

Резюме

Еднократно приложение на индуктора на интерферон дипиридамола демонстрира отчетлив антивирусен ефект въвеждан интраперитонеално или *per os* в мишки с експериментално индуцирана алфавирусна инфекция – енцефалит след инокулиране на Semliki Forest virus, прилаган *per os* у мишки с респираторна инфекция с причинители грипни вируси A/Puerto Rico/8/34 (H1N1) и B/Lee/40, прилаган интраперитонеално или орално в мишки с енцефалит, индуциран с щамове Lennett или Ленинград 2 на вирус херпес симплекс тип 1, както и при кожна херпесна инфекция в мишки с щам Ленинград 2. При експерименталните енцефалити и респираторните инфекции антивирусната активност бе регистрирана чрез индекса на протекция – намаление на процента на кумулативния леталитет и чрез удължаване на средното време на преживяване. При херпесния дерматит бе отчетено подтискане развитието на симптоматиката.

Introduction

Tonew and Dzeguze (1977), M. Tonew *et al.* (1977) and E. Tonew *et al.* (1982) reported about the antiviral activity of pyrimido[5,4-d]-pyrimidine (dipyridamole, DP) when tested *in vitro* against a wide range of RNA- and DNA- containing viruses. These findings were confirmed and extended by *in vitro* studies on the antiviral effect of this compound carried out by Oehring and Schmidt (1978), Bankowski *et al.* (1981), Korbecki *et al.* (1985), partially by Snoek *et al.* (1994).

A new direction on the study of DP as an antiviral substance was traced after our communications (Galabov and Mastikova, 1980, 1982; Galabov *et al.*, 1985) that this substance is an interferon (IFN) inducer. We suggested that the antiviral activity of DP could be entirely due to its IFN-inducing capacity. This effect was particularly well expressed in experiments *in vivo* involving oral administration of the compound to mice, and in investigations in human volunteers (Galabov and Mastikova, 1982, 1983a,b; Galabov *et al.*, 1987; Grigoryan *et al.*, 1989; Konstantinov *et al.*, 1989).

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Our further studies were directed towards *in vivo* testing the efficacy of DP as an antiviral agent when administered as an IFN inducer, namely, (a) prophylactic single-dose administration (e.g. 24 h prior to virus inoculation or in the latency period of the infection), and (b) administration of DP at IFN-inducing doses.

The antiviral effect of DP was tested in four different models of experimental viral infections in mice infected with: (a) Semliki Forest virus (SFV) causing encephalitis; (b) influenza viruses A/Puerto Rico/8/34 (H1N1) and B/Lee; (c) herpes simplex virus (HSV) type 1, strains Lennet and Leningrad 2 encephalitis; (d) HSV type 1, Leningrad 2 strain skin infection.

Materials and Methods

Compound

2,6-(Dietanolamino)-4,8-dipiperidino-pyrimido-(5,4-d)pyrimidine (dipyridamole, DP). For *in vivo* experiments Antistenocardin 2 ml ampouls, 0.5% solution (Pharmachim, Sofia) was used. The working dilutions were prepared in a solvent consisting of tartaric acid 0.2%, polyethylene glycole 600 5%, distilled water to 100%. This solvent was used as placebo.

Test animals

Random-bred line H or Swiss albino mice, as well as inbred line C-57/Black mice.

Viruses

Semliki Forest virus (SFV) was cultivated via passages in primary cell culture of chick embryo cells and 2-3 intracerebral passages in newborn albino mice. Working virus represented 10% brain suspension. Influenza viruses A/Puerto Rico/8/34 (H1N1) and B/Lee/40 were subjected to serial intranasal passages in albino mice. The stock virus represented lung suspension with an infectious titre of $10^{4.3}$ LD₅₀/ml and $10^{5.5}$ LD₅₀/ml, respectively. Virus herpes simplex (HSV) type 1, strains Lennet and Leningrad 2, underwent serial passages in human embryonal lung diploid fibroblasts.

Experimental infection with Semliki Forest virus in albino mice

Albino mice of the random-bred line H, weighing 10-12 g (15-20 animals per test group) were infected intraperitoneally (i. p.) with 10 or 100 MLD₅₀ of SFV. DP was administered singly, i. p. or *per os* 24 hours before, 24 or 72 hours after virus inoculation. The observation period was 30 days. The cumulative mortality percentage, with evaluation of the protection coefficient (PC) and the protection index (PI), and the MST in the treated and the placebo groups were determined.

Experimental infections in mice with influenza viruses A and B

Albino mice with a body weight of 10-12 g of the random-bred line H (15 animals per test group) were infected intranasally with influenza virus A/Puerto Rico/8/34 (H1N1) or B/Lee/40 (under ether anesthesia). DP was administered singly orally 24 h prior to, 24 or 72 h post virus inoculation. The observation period was 15 days. The cumulative mortality percentage and MST in the treated and placebo groups was recorded.

Infection with HSV type 1 (experimental herpetic encephalitis) in albino mice

Albino mice (15-20 animals per test group) were infected i. p. with 10 or 100 MLD₅₀ of HSV-1 strains Lennett or Leningrad 2. DP was administered singly i. p. or *per os* 24 hours before, 24 or 72 hours post virus inoculation. Observation period was 30 days. The cumulative mortality percentage and the MST in the tests and the placebo groups were checked.

Experimental HSV-1 induced skin infection

Mice C57/Black weighing 20 g (14 animals per test group) were infected via rubbing of undiluted tissue culture virus with a titre of $10^{6.33}$ TCID₅₀ in the scarified preliminarily de-haired skin on the mouse back. DP was administered singly i. p. 24 hours before, 72 hours or 120 hours post virus inoculation. The chronology of the appearance and the development of the local herpetic lesions in the test and the placebo groups were recorded.

Statistical treatment and analysis of the experimental data

The testing of DP antiviral activity *in vivo* the protection coefficient (PC) and the protection index (PI), as well as the MST were evaluated.

PC = mortality % in the placebo group / mortality % in the DP treated group

$$PI = PC - 1 / PC \times 100$$

The statistical treatment of the mortality data included determination of (a) the mean error (m_p) according to the formula

$$m_p = \pm \sqrt{p(100-p) / n-1},$$

where **p** is the mortality percentage,

n – number of animals per test group

and (b) by the values of t, evaluated according to Ilyenko (1977).

Results

Effect of DP in experimental alphavirus (SFV) infection

DP was administered i. p. 24 h before virus inoculation with single doses of 0.1, 1.8, 5.5 and 16.7 mg/kg, a dose range corresponding to the IFN-

inducing doses of this substance in albino mice. In Fig. 1A it is seen the effect of this compound during infection with 10 LD₅₀ of SFV, corresponding to approximately LD₉₅. DP at a dose of 0.1 mg/kg reduces mortality to 66.7%. With higher doses, the effect was slightly increased, reaching a maximum at 5.5 and 16.7 mg/kg - 50% of the mice survived and the mean survival time (MST) was extended by 9.7 days.

The protective effect was more strongly pronounced in infection with 100 LD₅₀ (Fig. 1B). When DP was administered in doses of 5.5 and 16.7 mg/kg the protection rates were 46.7% and 50%, respectively, and a prolongation to 19.5 days in the MST was observed, while the MST in the placebo group was 7.6 days

Oral administration of DP resulted in an even better expressed antiviral effect. The results fully correlated with the stronger IFN-inducing activity

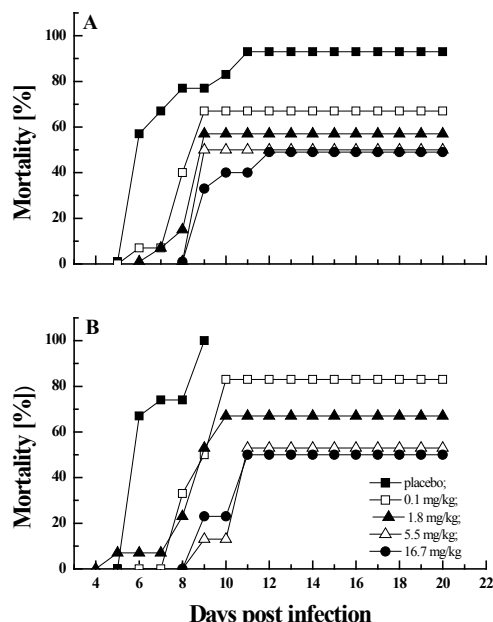


Fig. 1. Effect of DP in mice infected with alphavirus SFV: i. p. single administration 24 h before virus inoculation by 10 MLD₅₀ (A) and 100 MLD₅₀ (B)

of DP when administered per os. In infection with 5-10 LD₅₀, the antiviral effect of a single dose of DP depends on the time of its administration. When administered 24 h prior to infection, the optimal IFN-inducing doses, 12.5 and 25 mg/kg, were most effective (Fig. 2A). The percentage of surviving animals was 93% and 80%, respectively (70% mortality in the placebo group) and the MST was prolonged by 12.2 and 9.5 days, respectively. The dose-response curve retained its character when DP was administered 24 h after virus inoculation, although in that case the antiviral effect was noticeably reduced (Fig. 2B).

Fig. 2C shows the effect of the compound applied at the 72nd h after infection, i.e. at the middle of the latency period. It is noteworthy that DP in this case showed also marked efficiency, at doses of 50 and 100 mg/kg the protective rate being 50% and 80%, respectively, and the lengthening of the MST – 6.5 and 10.9 days. The dose of 12.5 mg/kg was found to be inactive. This unexpected result could be explained with some peculiarities of the development of the alphavirus infection and possibly by the more rapid attainment of the efficient IFN inducer concentration.

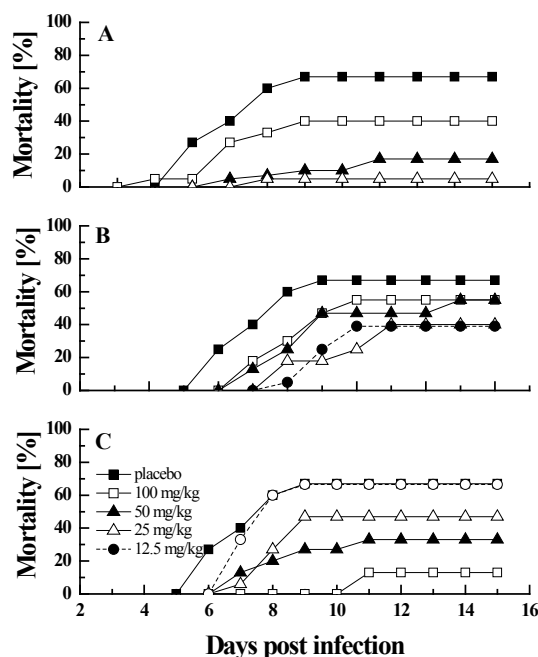


Fig. 2. Effect of DP in mice infected with alphavirus SFV: oral single administration of DP at virus infection with 10 MLD₅₀ – 14 h prior (A), 24 h post (B) or 72 h post virus inoculation (C)

Effect of DP on experimental infections with influenza viruses A and B

Mice inoculated intranasally by 5-10 LD₅₀ of influenza virus A/Puerto Rico/8/34 (H1N1) were treated *per os* with single DP doses – 100, 50 or 12.5 mg/kg 24 h prior to, 24 or 72 h post virus inoculation. We found a marked protective effect when the substance was applied 24 or 72 h after virus inoculation, and no distinct effect was registered when DP was administered prior to virus inoculation (Figures 3A, 3B, 3C).

This protective effect was approximately equal to that observed by Tonew et al. (1982) when DP was applied by oral route 5 times (-2, +2, +24, +48 and +72 h) at doses of 40 mg/kg, or 80 mg/kg (the first one divided in two daily intervals) in mice infected with influenza virus A/England/42/72 (H3N2).

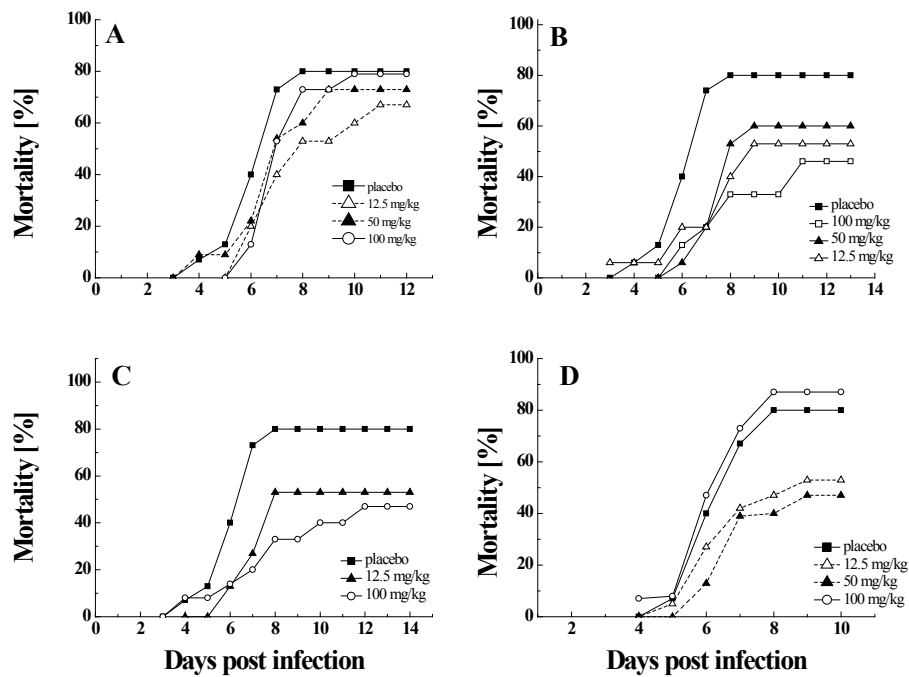


Fig. 3. Effect of DP in mice infected with influenza virus A/Puerto Rico/8/34 (H1N1), administered singly *per os* 24 h prior to (A), 24 post (B) or 72 h post (C) virus inoculation (5-10 MLD₅₀). DP applied orally 24 h post infection with influenza virus B/Lee/40 (D).

DP also exhibited a protective effect in albino mice experimentally infected with influenza B/Lee/40, 10 LD₅₀, when administered 24 h after virus inoculation at the IFN-inducible concentration of 12.5-50 mg/kg (Fig. 3D). In this case, the substance was inactive when administered 72 h post virus inoculation, while a single dose administered 24 h prior to viral inoculation in a dose of 100 mg/kg resulted only in an extension of the survival time by 1.6 days.

Effect of DP in experimental HSV type 1-induced encephalitis

After single-dose i. p. administration of DP 24 h prior to HSV type 1/strain Lennett infection, DP was effective only at the optimal IFN-inducing dose of 16.7 mg/kg. The protective rate was 44.7% and the prolongation of the MST from 6.3 days in the placebo group attained 17.3 days in the DP treated group (Fig. 4A).

In experimental infection with HSV type 1/strain Leningrad 2, a certain protective effect was found only in a 50 mg/kg DP dose administered 24 h prior to virus inoculation. The effect at 16.7 mg/kg was not significant (Fig. 4B). Applied i. p. post virus inoculation, DP was ineffective (Fig. 4C).

Following single-dose oral administration of DP 24 h before and 24 or 72 h post virus inoculation (Leningrad 2 strain) (Fig. 5), a distinct protective effect was observed in a dose of 12.5 mg/kg (optimal IFN-inducing dose) - a decrease of

mortality and prolongation of the MST by 4.7 days (when administered 24 h before infection) and 4 days (applied 24 h post infection), respectively, as well as certain delay of the MST by 7.5, 5.0 or 7.1 days (after administration 24 h prior to, and 24 or 72 h after virus inoculation, respectively).

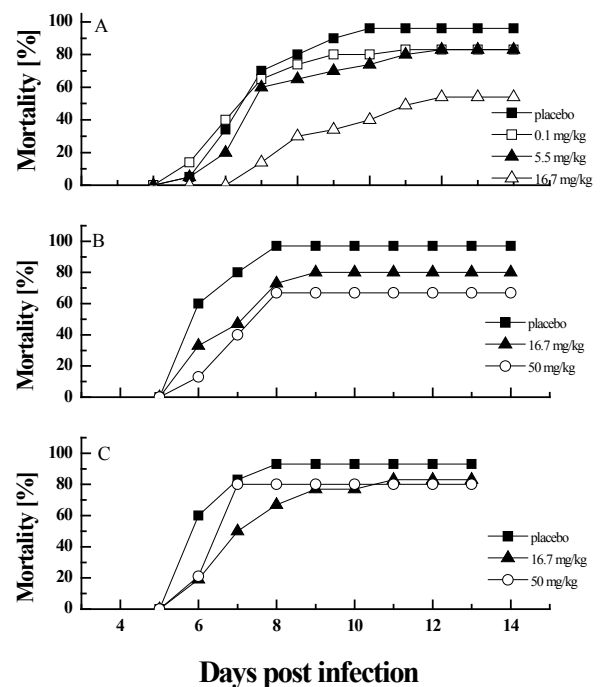


Fig. 4. Effect of DP single i. p. administration in mice with HSV type 1 (10 MLD₅₀) -induced encephalitis: DP applied i. p. 24 h prior to (A) HSV-1 Lennett strain infection, (B) 24 h prior to HSV-1 Leningrad 2 strain virus infection; (C) 24 h after HSV-1 Leningrad strain virus inoculation

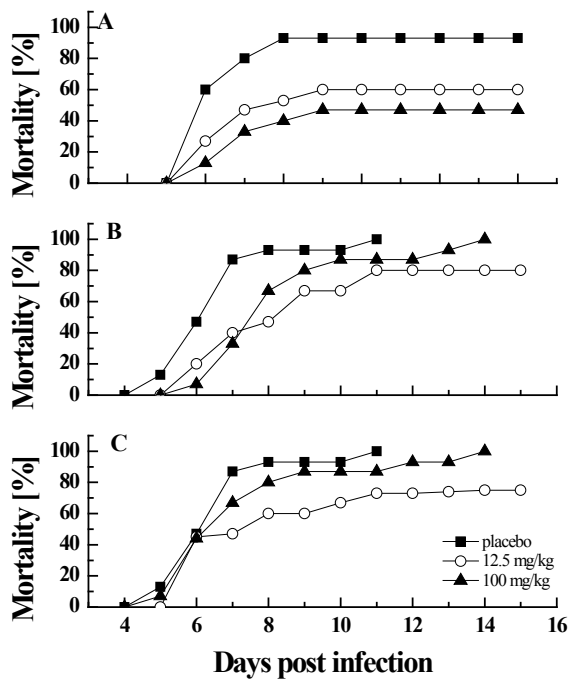


Fig. 5. Effect of DP single oral administration in mice with with HSV type 1 strtain Leningrad 2 (10 MLD50) – induced encephalitis: 24 h before (A), 24 h after (B) or 72 h post (C) virus inoculation

Effect of DP in experimental cutaneous infection with HSV type 1

Mice of C57Bl line, with a 5-day latency

period from the 6th to the 10th day post infection with HSV type 1 strain Leningrad 2, developed a typical picture of cutaneous herpes simplex (Table 1). DP single i. p. administration of a single 50-mg/kg dose suppressed markedly the course of herpes simplex infection if the compound was introduced 24 h before or 72 h post virus inoculation. The development of local lesions (vesicles and crusts) was prevented in 37.7% of the animals treated, whereas in the placebo group only in 7.1% local lesions were not demonstrable. In the ill animals treated with DP, the infection had a weaker course and on the 16th day a recovery in all animals was recorded, while in the placebo group – in 42.9% of the animals only. Following IFN inducing administration, at the 72nd h a stronger antiviral effect in comparison with the experimental group treated 24 h prior to infection was observed. Even at the 8th day a recovery was recorded in 2/3 of the animals with manifested skin herpes, i.e. animals with no lesions were in sum 71.4% of the total number of mice, 35.7% being in the group treated 24 h before virus inoculation, and zero percentage in the placebo group.

DP applied in the 120th hour, i.e. at the end of the latency period did not influence the course of infection.

Table 1. Effect of dipyrindamole (i.p. administration of a single daily dose of 50 mg/kg) on skin infection with herpes simplex virus type 1 (Leningrad 2 strain) in mice (C57BL)

Time of DP* administration	Skin lesions development (*total mice number)														
	Days post virus inoculation														
	6th			7th			8th			9th			10th		
	+V	+Cr	n.l.	+V	+Cr	n.l.	+V	+Cr	n.l.	+V	+Cr	n.l.	+V	+Cr	n.l.
Placebo	100.0	0	0	100.0	0	0	76.9	23.1	0	69.3	30.7	0	0	53.8	46.2
-24 hr	0	35.7	41.7	16.6	41.7	41.7	16.6	41.7	41.7	16.6	41.7	0	0	100.0	64.3
+72 hr	0	35.7	0	23.1	76.9	0	23.1	76.9	0	23.1	76.9	0	0	100.0	64.3
+120 hr	100.0	0	0	100.0	0	0	63.6	36.4	0	45.5	54.5	0	0	33.3	66.7

*DP = dipyrindamole; V = vesicles; Cr = crusts; n.l. = no lesions

Discussion and Conclusions

The results obtained from testing the antiviral activity of DP in experimental viral infections in mice indicate that this IFN inducer has manifested a pronounced protective effect by decreasing mortality and prolonging the MST. The effect was particularly well expressed in experimental infection with SFV in mice. DP was applied according to the IFN inducer scheme in single oral administration at the optimal IFN-inducing dose 24 h prior to lethal viral infection with SFV, 10-100

LD₅₀. In this mode of administration, DP reduced several times the mortality of infected animals. A protective effect, though weaker, was found with single-dose administration 24 and 72 h after viral inoculation. A noticeable protective effect was also observed after i. p. administration at optimal IFN-inducing dose.

In experimental orthomyxovirus infection in mice (influenza A/PR8/34 (H1N1) and strain B/Lee/40) DP was most effective when administered 24 h after inoculation (10 LD₅₀), being lless effective

24 h prior to infection, and ineffective 72 h post infection. Our results are approximately the same as the results obtained by Tonew *et al.* (1982).

The identity of single- and multiple-dose effects of DP administration in influenza infections supports our argument that the antiviral effect of DP is due to its IFN-inducing activity.

A prominent protective effect occurs in experimental brain infection with HSV type 1 when DP is administered 24 h before viral inoculation (i. p. or oral route of administration). Later administration of the preparation reduces that effect. DP has a distinct protective effect in experimental cutaneous infection with HSV type 1, strain Leningrad 2, in albino mice when administered once 24 h before and 72 h after viral inoculation.

The antiviral effect of DP is due to its IFN-inducing activity. The characteristics of the IFN-inducing activity of DP *in vitro* and *in vivo* are the most convincing argumentation in this respect. The effective scheme in experimental viral infections is single-dose administration of DP at optimal IFN-inducing doses. A similar correlation between IFN-inducing and antiviral activity exists in other low-molecular-weight IFN inducers. IFN-induced DP could also affect the immune system (Koychev *et al.*, 1985; Mihaylova *et al.*, 1985). It is quite likely that, by stimulating the lymphoreticular system for IFN production, the substance actually stimulates other components of the immune system. Obviously, the protective effect of IFN may also include the effect it exerts on other systems, but that would require further detailed studies of its impact on the immune system.

The conclusions regarding the antiviral activity of DP in experimental viral infections in white mice are as follows:

(1) DP is an effective antiviral agent in experimental viral infections with SFV, HSV type 1, influenza A/Puerto Rico/8/34 (H1N1) and influenza virus B (Lee strain).

(2) The compound shows higher antiviral activity with a single administration at IFN-inducing doses: from 12.5 mg/kg to 100 mg/kg by oral administration, and from 5.5 mg/kg to 50 mg/kg by i. p. route of administration.

(3) The antiviral effect of DP is most clearly expressed when administered 24 h before, and 24 h and 72 h after viral inoculation in experimental infections with SFV and HSV type 1, at infection with influenza A(H1N1) - 24 h or 72 h post-infection, and in influenza B/Lee - 24 h after infection.

These data are new proofs of the hypothesis that the antiviral effect of DP is due to its IFN-inducing capacity. Besides, these results may serve as a basis for determination of the optimal scheme of treatment by DP in the clinics.

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Molecular Biological Characteristics of Bovine Gamma Herpesvirus 4 Originated from Different Clinical Sources

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Abstract

Bovine herpesvirus 4 (BoHV 4) is a member of the *Herpesviridae* family in the *Gammaherpesvirinae* subfamily, *Radinovirus* genus. The purpose of this study was to isolate BoHV 4 from calves, heifers and cows presenting various clinical entities – respiratory, conjunctivitis, pneumonia, skin damage, genital lesions and abortion, and to characterize its molecular biological features.

Primary and permanent bovine cell cultures were used for virus isolation and culturing. Polymerase chain reaction (PCR) was used to confirm the gB and TK genes and the PCR products were sequenced.

After infecting the primary bovine and permanent cell lines, a cytopathic effect typical of BoHV 4 was observed and 10 cytopathic viral agents were isolated. Amplification products with molecular weights of 567 and 615 bp for the Tk and gB genes, respectively, were found after PCR.

After the BoHV 4 isolates had been sequenced, 473 and 476 nucleotide sequences were analyzed for the Tk and gB genes, respectively. The phylogenetic trees constructed based on the Tk and gB genes of Bulgarian BoHV 4 strains fall within the same branch with the reference BoHV 4 European strains deposited in the genomic bank and their genomic features were similar to European sequenced genomes of other *Radinoviruses*.

Keywords: BoHV 4, PCR, sequencing

Резюме

Говеждият херпесвирус 4 (BoHV 4) е член на семейство *Herpesviridae* в подсемейство *Gammaherpesvirinae*, род *Radinovirus*. Целта на това изследване е да се изолират BoHV 4 от телета, юници и крави с различни клинични симптоми - респираторни, конюнктивити, пневмонии, увреждания на кожата, генитални лезии и аборти, и да се охарактеризират неговите молекулярно-биологични особености.

За изолиране и култивиране на вируса са използвани първични и постоянни култури от говежди клетки. За потвърждаване на gB и TK гените е използвана полимеразна верижна реакция (PCR) и продуктите от PCR са секвенирани.

След инфектиране на първичната говежди и постоянни клетъчни линии е наблюдаван цитопатичен ефект, типичен за BoHV 4 и са изолирани 10 цитопатични вирусни агенти. След PCR са получени амплификационни продукти с молекулно тегло 567 и 615 bp за Tk и gB гените съответно.

След секвениране на BoHV 4 изолатите са анализирани 473 и 476 нуклеотидни последователности за Tk и gB гени. Конструираните филогенетични дървета на базата на Tk и gB гена на български щамове BoHV 4 попадат в един клон с референтните европейските BoHV 4 щамове, депозирани в геномната банка и техните геномни характеристики са подобни на геномите на секвенираните европейски радиновируси.

Introduction

Bovine herpesvirus 4 (BoHV 4) now named as Bovine gammaherpesvirus 4 is a member of the *Herpesviridae* family in the *Gammaherpesvirinae* subfamily, *Radinovirus* genus (Davison *et al.*, 2009).

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The BoHV 4 genome is small with type B structure characterizing the subfamily *Gammaherpesvirinae* (Roizman and Pellet, 2001). It has a collinear arrangement of double-stranded nucleic acid (DNA) with a size of 144 ± 6 kb. BoHV 4 genome is organized into a long, unique segment (long unique region - LUR) with a size of 108 ± 2 kb and two repetitive double tandem sections non-coding sequences rich in guanine and cytosine (G + C), which make up the polyrepetitive (pr) DNA (pr DNA), with a size of 1450 to 3050 bp (Bublot *et al.*, 1991; Broll *et al.*, 1999). BoHV 4 contains a gene encoding thymidine kinase (TK) (Ehlers *et al.*, 1985; Kit *et al.*, 1986; Thiry *et al.*, 1989; Bublot *et al.*, 1992; Lomonte *et al.*, 1996; Zimmermann *et al.*, 2001).

Based on the restrictase fragment analysis (RFA), BoHV 4 isolates are classified into two genome types - 1) similar to the European strain Movar 33/63 and 2) similar to the American strain DN 599 (Thiry *et al.*, 1989; Bublot *et al.*, 1991). Another classification divides the BHV 4 strains into 3 categories: 1) isolates similar to strain DN 599, 2) similar to strain Movar 33/63 and 3) unclassified strains having a specific restrictase fragment pattern (Thiry *et al.*, 1989; 1990; Bublot *et al.*, 1991).

BoHV 4 was isolated from calves and heifers with different clinical entities - respiratory symptoms and keratoconjunctivitis (Bartha *et al.*, 1966; Mohanty *et al.*, 1971), bovine pneumonia (Smith *et al.*, 1972) endometritis, abortion, enteritis, skin damage, skin nodules, pustulous mammilitis, breast ulcers, interdigital dermatitis (Reed *et al.*, 1977; Cavarani *et al.*, 1990, 1996; Donofrio *et al.*, 2000) and animals with nodular dermatitis (House *et al.*, 1990).

The purpose of this study was to isolate BoHV 4 from ruminants with different clinical symptoms and to characterize its molecular biological features.

Materials and Methods

BHV 4 virus isolation and cultivation

Parts of internal organs (lungs, liver, spleen, kidneys, lymph nodes, uterus, mucous membrane, intestines, tongue epithelium, brain, spleen, kidneys from aborted fetuses, placental cotyledons, and buffy coat) from bovine animals with various clinical signs (respiratory, genital and abortion) were used for virus isolation. The organ tissues were homogenized to 10% (w/v) suspension in cell culture medium, and then were centrifuged at $1500 \times g$ for 10 min. The supernatants were filtered through 0.45 μm -pore-size millipore filters and used

to infect the cell cultures. Nasal and ocular swabs were placed in cell culture medium, centrifuged at $1500 \times g$ for 10 min and clear supernatants were used to inoculate the cell monolayers.

Primary bovine kidney, testes and permanent cell cultures from embryonal bovine trachea (EBTR) were used for virus isolation and culturing. As growing medium, Eagle's minimal essential medium (E MEM) was used with 10% fetal calf serum (FCS), 0.075% sodium bicarbonate, 20 mM HEPES buffer, 0.2M-L glutamine, and antibiotics, penicillin 100 UI/mL, and streptomycin 100 μg /mL. The same medium with 3% FCS was used as maintenance medium. Infectious titers were calculated using the Reed and Muench method (1938).

Molecular biological investigation

Polymerase chain reaction (PCR) for demonstration of BoHV 4 Tk and gB genes

Amplification of the Tk gene was performed by conventional and nested Tk-PCR, (Egyed *et al.*, 1996) with some modifications (the DNA matrix in the classical reaction was amplified by PCR mix Fideli Tag PCR Master Mix (2X), England).

Primers selected from the sequences of the BoHV 4 Tk gene were used for amplification (Lomonte *et al.*, 1992). Sequences were selected from the National Center for Biotechnology Information (NCBI Gene Bank) database (AC 49773) (Table 1). Outer primers Tk 1 and Tk 2 were used for the construction of 567-base pair fragment. The internal or nested set of primers Tk 3 and Tk 4 were used for the formation of 260 bp product. Amplification of the Tk gene was performed in a QB-96 (UK) thermal cycler in a volume of 25 μL , with amount of DNA between 0.2 and 0.4 $\mu\text{g}/\mu\text{L}$, 10 pmol of each primer, Fideli Tag PCR Master Mix (2X), and distilled water to 25 μL . In the first PCR round, amplification started with pre-denaturation at 95°C for 10 min, followed by 30 cycles at 94°C for 60 sec, at 60°C for 60 sec and at 72°C for 90 sec and a final extension of 7 min at 72°C . A 5 μL volume of the PCR product from the first PCR round was further amplified by a nested PCR round using the same PCR mix as described for the first round but with the nested set of primers Tk 3 and Tk 4 (Table 1).

Analysis of PCR products was carried out by horizontal agarose (1%) DNA gel electrophoresis in 0.5X Tris, Boric acid, EDTA buffer (TBE) buffer for 1 h at 120 V. Gels were stained with 1 μg /mL ethidium bromide and photographed under ultraviolet light.

Table 1. Data for used primers and their nucleotide position for amplification of BHV 4 DNA

Oligo nucleotides	DNA sequencing	Nucleotide position of the primers	PCR products [bp]
Tk 1	5'-GTTGGGCGTCCTGTATGGTAGC-3'	132 - 153	567
Tk 2	5'ATGTATGCCCAAACTTATAATATGACCAG-3'	669 - 698	567
Tk 3	5'-TTGATAGTGCCTTGTGGGATGTGG-3'	339 - 363	260
Tk 4	5'-CACTGCCCCGGTGGGAAATAGCA-3'	577 - 598	260
gB-1	5'-CCCTTCTTTACCACCACCTACA-3'	38 - 17	615
gB-2	5'-TGCCATAGCAGAGAAACAATGA-3'	555 - 576	615

PCR described by Wellenberg *et al.* (2001) with modifications (Fideli Tag DNA PCR Master Mix (2X), England, used for the conventional reaction) was used for the amplification of the gB gene.

Primers gB1 and gB2 exhibiting 100% homology to the sense chain at nucleotide positions 38 to 17 (gB 1) and the opposite nucleotide chain at positions 555 to 576 (gB 2) of the gB gene sequence of BHV 4 were used (Goltz *et al.*, 1994) (Table 1).

Amplification was performed in QB 96 Thermal cycler (UK). The program began with pre-denaturation at 95° C for 10 min, followed by 45 cycles with parameters: 94° C for 10 sec, 58° C for 60 sec and 72° C for 90 sec and a final extension of 72° C for 7 min.

DNA derived from other herpes viruses - bovine herpes virus 1 (BoHV 1) Pchelarov, goat herpes virus 1 (GHV 1) Trojan, swine herpes virus 1 (SHV 1) A 2, and EBTR cell culture uninfected with BoHV 4 were used to determine the specificity of the reactions. European strains Movar 33/63, M52 BP 90, M52 BP 10, M51 MZ 80 and the American strain DN 599 were used as reference BoHV 4 strains.

Sequencing of BHV 4 Tk and gB genes

For the sequencing of the Tk and gB genes, Bulgarian BoHV 4 strains Levski, Nikolovo, Svoboda, Kazichene, Abomasum heifer 1 Godech, Brain cow 1 Godech, Brain cow 4, Brain cow 3053 and the Hungarian strains Movar 33/63, M52 BP 90, M51 MZ, and M51 MZ 80 were used. Sequencing was performed after multiplication of portions of Tk and gB genes using specific primers (Table 1) giving the product of 567 bp for the Tk and 615 bp for gB genes, respectively. For amplification of the Tk and gB genes, the PCR programs described above were used. The amplicons obtained were purified through S-400 columns and a DNA gel purification kit (GE Healthcare, UK). Purity and amounts of the products were determined spectrophotometrically

(Gene Quant II, Pharmacia LKB, UK) or after gel electrophoresis in 1% agarose gel. The amplified products were sequenced twice with each of the specific primers mentioned above.

Sequencing was performed in a MegaBACE Sequencer 1000 (Amersham, Bioscience, USA) with the DYEnamic ET Dye Terminator Cycle Sequencing Kit (GE Healthcare, UK) according to the manufacturer's instructions.

The data obtained from the nucleotide and amino acid sequences were used to construct the phylogenetic trees. The multiple alignments of the amino-acid and nucleotide sequences were performed with MUSCLE software (Edgar, 2004). The phylogenetic trees were constructed with JModelTest 0.1.1 (Posada, 2008) and ProtTest 2.4 (Abascal, 2005). For the building of the phylogenetic trees and their graphical representations, MEGA 7 software (Kumar *et al.*, 2016) and 1000 bootstrap replications were used.

The data obtained after sequencing were compared to the sequences of BoHV 4 gB gene from strains AJ 617688.1, AJ 617687.1, Movar 33/63, Z 15044.1 All Genes, EU 055543.1, JX 644989.1, MG 264399.1 USA Calif., MG 264397.1 USA Calif., MG 264404.1 USA Calif., KP209025.1 Aregn.PLOS, MH 605274.1 Turkey, JX 644988.1, KP 209016.1 Argentina, KP 209021.1 Argentina, KU 180390.1 Argentina, GQ 375280.1, GQ 246866.1 and GQ 246863.1 deposited in NCBI. The sequences of BHV 4 Tk gene of deposited strains AB035515.1, AB035517, AB035516, AF 318573, JN 133502, KC 999113, KX 355279 and S 49773 were used for comparison to the Bulgarian BoHV 4 strains.

Results

BoHV 4 virus isolation and cultivation

After the primary bovine and EBTR permanent cell lines had been infected with 10% suspension from the internal organs of animals with various clinical manifestations (respiratory,

genital and abortion) of the BoHV 4 infection, 10 cytopathic viral agents were isolated (Levski, Nikolovo, Svoboda, Kasichene, Momchilgrad, Lungs cow Godech, Brain cow 1 Godech, Brain cow 4 Godech, Brain cow 3053 Godech and Abomasum heifer 1 Godech).

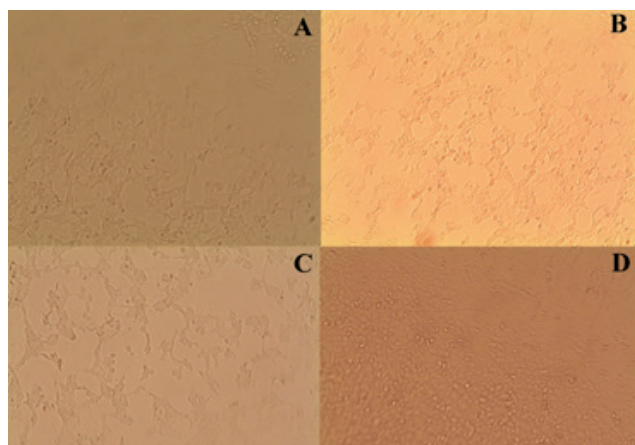


Fig. 1. Cytopathic effect at 48th hours (A), 72nd hours (B), and 120th hours (C) on EBTR cells incubated with 10% organ suspensions from the heifer Godech with characteristic for BoHV 4 clinical symptoms. Control uninfected EBTR cell culture (D). Magnification X 100.

Cytopathic changes characterized by rounding and tearing of the cell monolayer were observed at 24 – 48th h after inoculation. The changes were amplified at 72nd h and after 120th h the cell monolayers were disrupted completely (Fig. 1 B and C). The cytopathic changes of the cell monolayer were not observed in control, non-infected cell cultures (Fig. 1 D).

Molecular biological investigation

Polymerase chain reaction (PCR) for demonstration of the BoHV 4 Tk and gB genes

Amplified products of the Tk gene were with molecular weight of 567 bp after the first PCR (Fig. 2, line 1 to 9). Amplicons of the other used isolates - BoHV 1 Pchelarovo (Fig. 2, line 10), GHV 1 Troyan and SHV 1 A 2 (data not shown) were not observed. Amplification products with a size of 260 bp were obtained after the nested PCR for all BHV 4 isolates investigated (data not shown).

Amplicons with a molecular weight of 615 bp corresponding to the size of the gB gene of BoHV 4 were obtained for all Bulgarian and reference isolates of BHV 4 after the multiplication of gB gene (Fig. 3, line 1 to 8). Amplified products of the gB gene were not observed in the other isolates used - BoHV 1 Pchelarovo (Fig. 3, line 9), GHV 1 Troyan and SHV 1 A 2 (data not shown).

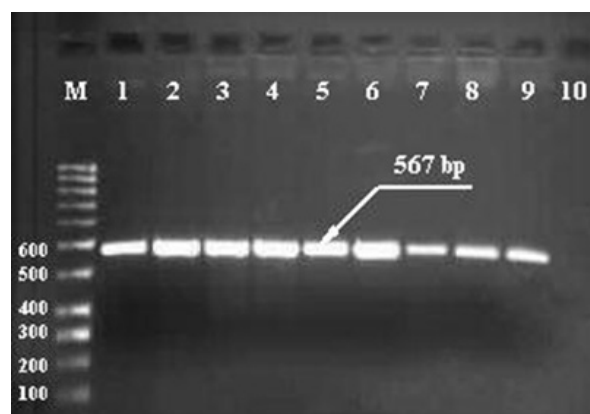


Fig. 2. Classical PCR for multiplication of Tk gene of isolated BoHV 4 strains after first PCR. Line M – molecular weight marker 100 bp, line 1 - “Nikolovo“, line 2 - “Kazichene“, line 3 - “Svoboda“, line 4 - “Momchilgrad“, line 5 - “Levski“, line 6 - “Lungs cow Godech“, line 7 - “DN 599“, line 8 - “Movar 33/63“, line 9 - “MZ 80“, line 10 - BoHV 1 “Pchelarovo”.

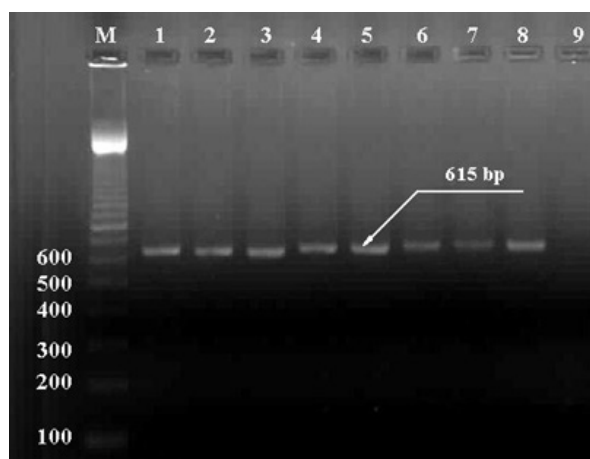


Fig. 3. Classical PCR for gB gene amplification of investigated BoHV 4 isolates. Line - M - Marker of molecular weights 100 bp, line 1 - “Nikolovo“, line 2 - “Kazichene“, line 3 - “Svoboda“, line 4 - “Momchilgrad“, , line 5 - “Lungs cow Godech“, line 6 - “DN 599“, line 7 - “Movar 33/63“, line 8 - “MZ 80“, line 9 - BoHV 1 “Pchelarovo”.

Sequencing of BHV 4 strains.

After the sequencing of the Tk gene of BoHV 4 isolates, 473 nucleotide sequences were analyzed.

Bulgarian isolates Levski, Nikolovo the reference AB 035517, AF035517 and KX355279-Turkey strains formed one branch with 54% homology of the phylogenetic tree based on sequencing of the Tk gene. Referent strains KC 999113 and AB 035515-B 11 formed separate clusters of this branch (with 54% homology). On a separate branch of the phylogenetic tree, with 43% homology were locat-

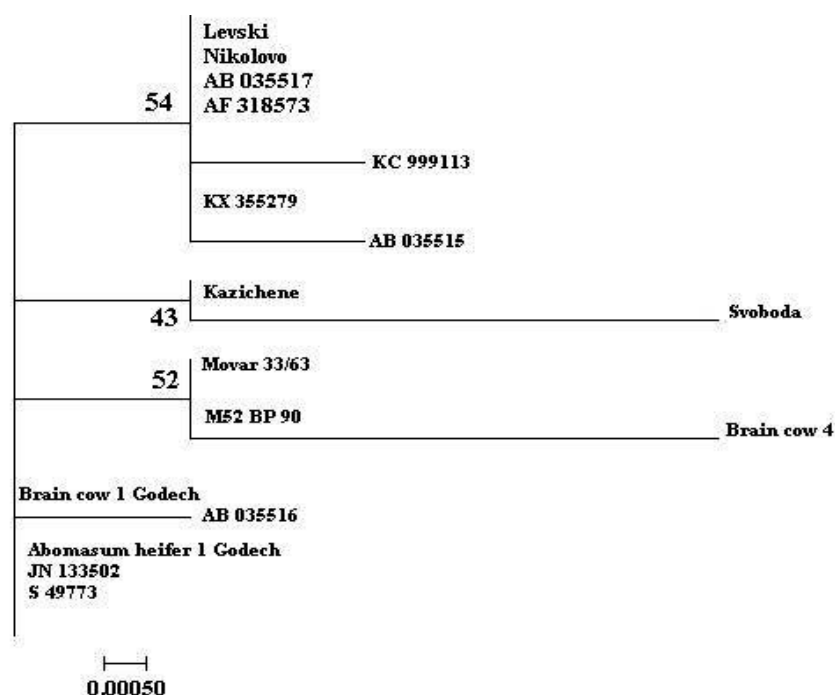


Fig. 4. Phylogenetic tree based on nucleotide sequences comparison from Tk gene of investigated Bulgarian and deposited in gene bank reference BoHV 4 strains.

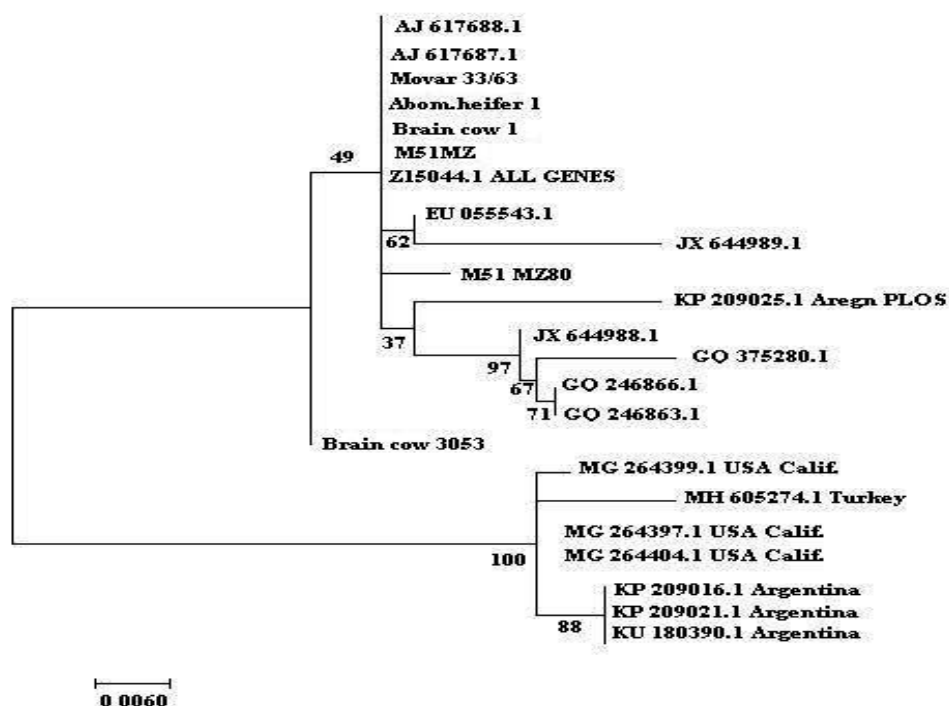


Fig. 5. Phylogenetic tree based on nucleotide sequences comparison from gB gene of investigated Bulgarian BoHV 4 strains and deposited in gene bank reference BoHV 4 strains.

ed strains Kazichene and Svoboda. Movar 33/63, M52 BP 90 and Brain cow 4 Godech were found to reside on the same branch with 52% homology. Strains Brain cow 1 Godech, Abomasum heifer 1 Godech, JN 133502-Belgium and S 49773 were located on the main branch of the phylogenetic tree. The reference strain AB 035516 formed a different clone of the tree (Fig. 4).

The analysis of 476 nucleotide sequences obtained from the gB gene was performed from base

518 to base 994, corresponding to the reference sequence.

After the construction of the phylogenetic tree based on sequencing of the gB gene, Bulgarian isolates Abomasum heifer 1 Godech, Brain cow 1 Godech as well as the European reference strain AJ 617688.1, AJ 617687.1, Movar 33/63, M51 Mz, and Z 15044.1 All Gene were shown to belong to the same branch of the phylogenetic tree with 49 % homology. Referent strains EU 055543.1 and

JX 644989.1 formed a separate cluster with 62% homology of this branch (with 49 % homology). Strains JX 644988.1, GQ 375280.1, GQ 246866.1 and GQ 246863.1 formed different branches of the phylogenetic tree with 97% homology. The Bulgarian strain Brain cattle 3053 did not belong to any branch of the tree. In different clones of the phylogenetic tree with 100% homology are the American, California MG 264399.1, MG 264397.1, MG 264404.1, Turkey MH605274.1, and the Argentinian strains KP 209016.1, KP 209021.1 KU 180390.1 with 88% homology (Fig. 5).

Discussion

After inoculation of primary and permanent EBTR cell cultures, ten cytopathic agents with typical culture characteristics for BoHV 4 were isolated. In the control uninfected cell culture cytopathic agents were not observed. The isolated viral agents were confirmed as BoHV 4 by PCR with specific primers multiplying BoHV 4 Tk and gB genes. It is known that replication and morphogenesis of BoHV 4 is different than that of the other bovine herpesviruses, which is confirmed by our investigation. The primary replication of BoHV 4 is performed in respiratory epithelial cells (Yang *et al.*, 2019). The visible cytopathic changes of newly isolated strains were slow and discrete and were observed after 48 h on EBTR cell culture. Subsequently, the propagation of BoHV 4 is increased, which is evidence of the typical slow accumulation of BoHV 4.

The isolated on primary and permanent cell cultures strains from brain, spleen, buffy coat, abomasum and lungs originating from cattle and calves from different country regions were confirmed by PCR for the gB and Tk of BoHV 4 genes.

The classical and nested PCR with specific primers for Tk gene (Lomonte *et al.*, 1992; Egyed *et al.*, 1996) allowed us to perform more accurate studies of the in vivo prevalence of BoHV 4 in the organs and the peripheral blood leukocytes of the diseased animals. The absence of DNA multiplication obtained from the heterologous herpesvirus strains confirms the specificity of PCR. Evidence that both reactions, classical and nested PCR, are highly sensitive is the lack of bands in the other isolates used - BoHV 1 Pchelarovo (Fig. 2, line 10 and Fig. 3, line 9), GHV 1 Troyan and SHV 1 A 2 (data not shown).

The gene encoding glycoprotein B is one of the most conservative genes among the Herpesviridae family members. Because of the fact that the primer's nucleotide sequences must be in the conserved region of the gB gene in order to prove all

BoHV 4 isolates, the choice of primers for performing the gB PCR is very important. The selected gB primers based on the oligonucleotide sequences of the gB gene (Goltz *et al.*, 1994) were specific as they have amplified DNA from all tested BoHV 4 strains and specific amplification for the other investigated animal herpesviruses was not observed.

The structure of the genotype of BoHV 4 is type B, similar to other Gamma herpesviruses (Roizman and Pellet, 2001). After the sequencing of the Tk gene of the Bulgarian BoHV 4 isolates, it was found that the isolates Levski and Nikolovo were grouped in the same branch with the reference BoHV 4 strains AB035517 and AB035515.1 deposited in the gene bank. BHV 4 strain isolated from Brain cow 4 Godech belonged to the same group together with the European reference strain Movar 33/63. Strains Kasichene and Svoboda are more distant in comparison with other Bulgarian strains and formed a different branch of the tree. The remaining sequenced Bulgarian strains Cow lungs Godech and Abomasum heifer No1 Godech did not fall into any of these groups. Most probably those isolates are phylogenetically more distant from the European reference strain or are closer to the American BoHV 4 strain. These results are evidence that the studied Bulgarian viral isolates are also BoHV 4. The genomic features of the sequenced Bulgarian isolates were similar to the sequenced genomes of other Radinoviruses (Broll *et al.*, 1999; Zimmermann *et al.*, 2001).

Conclusions

1. Bulgarian viral isolates originating from different clinical sources have molecular-biological characteristics typical of BoHV 4
2. The phylogenetic trees constructed based on the gB and Tk genes confirm that the Bulgarian BHV 4 strains have similar characteristics to the European referent BoHV 4 strains deposited in the genomic bank.
3. The amplification products obtained for the gB and Tk genes of Bulgarian BoHV 4 isolates after using specific primers allow more accurate in vivo studies of the presence of BoHV 4 in organs and peripheral blood leukocytes in animals.

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Impact of Growth Conditions on Biofilm Formation by Model Gram-Negative and Gram-Positive Bacterial Strains

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Abstract

Present-day medical microbiology faces the risks of biofilm-related infections. These are much more resistant to routinely applied antibacterial preparations, and are often the cause of recurrent infections. The search of novel anti-fouling preparations puts forward the elaboration of a reliable complex approach for selecting and estimating the successful agent. As a first step, screening procedures should include a fast methodology, and the crystal violet assay is widely applied for semi-quantitative estimation of biofilm biomass. However, the species- and strain-peculiar mechanisms of biofilm formation require optimisation of the experimental protocols with regards to the individual demands of model strains. This study aims to characterise the biofilm-forming behavior of a set of model Gram-positive and Gram-negative strains. The Gram-positive strains included in the experiments were *Staphylococcus aureus* ATCC 29213 and *Bacillus subtilis* 168. Gram-negative strains were *Pseudomonas aeruginosa* PAO1 and six *Escherichia coli* K-12 strains. The tested variables were growth medium, time of cultivation and growth temperature. Notably, these variables have different, sometimes even opposite effects on planktonic growth and biofilm development. The results provide information needed for the proper design of further screening experiments on these strains, for novel substances with putative anti-biofilm activities.

Keywords: Biofilm formation, Gram-positive and Gram-negative strains, effects of growth medium, temporal characteristics and temperature of cultivation

Резюме

Съвременната медицинска микробиология е изправена пред риска от инфекции, свързани с образуването на биофилми. Биофилмите са много по-резистентни към антибиотици и често са източник на рецидивирани инфекции. Търсенето на нови анти-биофилмни препарати поставя въпроса за създаването на комплексна методология за селекция и оценка на подходящи вещества. Първите стъпки следва да включват бърза скрининг-методология и тестът "кристал виолет" е един широко прилаган полу-количествен подход за оценка на биофилмната биомаса. Независимо от това, че този тест е широко приет, поради видово- и щамовоспецифичните механизми на биофилм образуването се налага оптимизиране на експерименталните протоколи с оглед индивидуалните изисквания на моделните щамове. Цел на изследването е да характеризира биофилм-образуващите характеристики на набор от моделни Грам-положителни и Грам-отрицателни щамове. Включените в изследването Грам-положителни щамове са *Staphylococcus aureus* ATCC 29213 и *Bacillus subtilis* 168. Грам-отрицателните щамове са *Pseudomonas aeruginosa* PAO1 и шест щамове *Escherichia coli* K-12. Изследвани са следните вариабилни: растежна среда, продължителност на култивирането и растежна температура. Тези фактори имат различен, понякога - дори противоположен ефект върху развитието на бактериите в течна среда и като биофилм. Резултатите дават възможност за създаване на подходящ експериментален дизайн за осъществяване на скрининг-изследвания върху тази група моделни щамове с включването на нови вещества с очаквана анти-биофилмна активност.

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Introduction

Bacterial biofilms represent cell communities embedded in extracellular polymer substances that grow attached to surfaces. Biofilm formation follows several stages: (1) reversible adherence of the bacteria to the surface; (2) loss of motility and irreversible attachment; (3) development of microcolonies; (4) differentiation of the mature biofilm; (5) detachment of motile bacteria from the biofilm and its dissemination to other loci (Souza dos Santos, 2018). Attachment is a complex process regulated by diverse characteristics of the growth medium, substratum, and cell surface (Donlan, 2002).

Since the initial thorough descriptions of the biofilms (Costerton *et al.*, 1994), it has further been well-recognised that sessile communities may develop on both environmental surfaces and on, or inside other organisms, including the human body (indwelling devices and tissues) where they cause biofilm infections (Wang *et al.*, 2015; Coughlan *et al.*, 2016). The latter are difficult to overcome, and are related with the recurrency of bacterial diseases (Vijayakumar *et al.*, 2016; Jamal *et al.*, 2018).

Biofilms may be considered an adaptive mode of life where sessile bacteria, much better than their free-floating counterparts, survive environmental hazards: toxic substances, desiccation, etc., or, inside plant or animal hosts – the evolutionary evolved immunity defences and/or antibacterial treatments (Jamal *et al.*, 2018). Therefore the development of novel effective anti-biofilm strategies is a serious challenge for researchers (Costerton *et al.*, 1994; Speranza *et al.*, 2018). As a first step of the estimation of putative anti-biofilm effects of novel substances, laboratory screening experiments are indispensable and these most often include the routinely applied crystal violet (CV) test. This is a reliable and easy-to-perform semi-quantitative assay for biofilm biomass. In screening experiments aimed to identify anti-biofilm substances, the CV assay can be applied to different model Gram-positive and Gram-negative microorganisms. While the test itself is well-known and easy to apply, in question is the standardization and/or optimization of the conditions for the biofilm growth of the model microorganisms prior to the CV staining.

For estimation of antibiotic sensitivity of non-biofilm bacteria, the growth media of choice and the respective protocols recommended by ISO are Mueller-Hinton broth and agar. Mueller-Hinton media provide satisfactory growth of most non-

fastidious pathogens, acceptable batch-to-batch reproducibility, low sulfonamide, trimethoprim, and tetracycline inhibitors and a large amount of data has been collected from antimicrobial susceptibility tests with this medium over several decades (<https://www.iso.org/obp/ui/#iso:std:iso:ts:16782:ed-1:v1:en>). Therefore these media are preferably applied also in screening protocols for novel antibacterials.

When choosing growth medium and growth conditions for experiments for anti-biofilm studies with biofilms the situation is however not so clear, mostly because of the complex nature of the sessile consortia. The necessity of careful elaboration of the experimental design in biofilm studies has been underlined by many researchers (Costerton *et al.*, 1994; Vijayakumar *et al.*, 2016; Speranza *et al.*, 2018), still present-day biofilm experiments are far from a standardized approach. The problem is the variable response of bacterial species and strains to the conditions of biofilm growth. Such a variety of responses is most probably due to the adaptive, convergent role of the biofilm for bacterial survival and the involvement of variable molecular mechanisms in the sessile growth. Our experience is that the outcome of anti-fouling experiments may depend, among other factors, on the bacterial strain and the nature of the explored substances (Vacheva *et al.*, 2011, 2012a; Borisova *et al.*, 2018). Hence, for proper experimental design a preceding thorough examination is needed of the sessile behavior of the chosen model strains. In literature, various cultivation protocols were applied by different authors prior to applying the CV dye. There are data obtained as a result of the application of different minimal media (among which M63) or rich media (among which LB). The medium-of-choice for antibiotic sensitivity estimation of non-biofilm bacteria, MH, is not quite popular in biofilm studies and has been used more rarely (e.g. Vijayakumar *et al.*, 2016).

Our team has lately been planning experiments for the identification of novel anti-biofilm substances of synthetic or natural origin. Bearing in mind the above considerations, the present study was undertaken with the aim to characterize the biofilm-formation behavior of a set of model Gram-positive and Gram-negative strains under variable experimental conditions. We have tested biofilm development in three media: M63, LB, and MH, and the impact of cultivation duration and growth temperature.

Materials and Methods

Bacterial strains and growth media

The Gram-positive strains included in the study were *Staphylococcus aureus* ATCC 29213 and *Bacillus subtilis* 168 (Vasileva-Tonkova *et al.*, 2011). *Escherichia coli* K-12 strains included W1655 F+, kindly provided by Prof. J. Gumpert, Institute of Molecular Biotechnology, Jena, Germany, strain 3110 (NCIPD, Sofia, Bulgaria) and strains 406, 409, 420 and 446 purchased from NBIMCC (Sofia, Bulgaria). More details on the K-12 strains are provided by Vacheva *et al.* (2012b). The *Pseudomonas aeruginosa* PAO1 strain was from the International Reference Panel (De Soyza *et al.*, 2013).

The growth media applied were: Tryptic soy agar (TSA), Tryptic soy broth (TSB) and Mueller-Hinton broth (MH) were purchased from Sigma-Aldrich. LB broth (10 g tryptone, 10 g NaCl and 5 g yeast extract per litre) and M63 medium (0.02 M KH₂PO₄, 0.04 M K₂HPO₄, 0.02 M (NH₄)₂SO₄, 0.1 mM MgSO₄ and 0.04 M glucose) were prepared in the laboratory.

Estimation of bacterial growth and biofilm formation

The bacteria were maintained as frozen stocks in TSB supplemented with 20% glycerol. Before the experiments, they were streaked on TSA for selection of single colonies. The strains were further applied on slant agar and kept as source of inocula for no more than 20 days. To prepare the inocula, a loopful of each strain was inoculated in TSB and cultivated overnight at 37°C. The overnight cultures were dissolved 1:100 in the test media (MH, M63, or LB), vortexed, and 150 µl quotes were applied into the wells of a 96-well plate, 6 wells per sample. The plates were incubated for 24 h at 37°C. To estimate the bacterial growth in each well, the optical density (OD) was first estimated by ELISA reader at 620 nm wavelength. Then the non-adherent bacteria were removed, the wells were washed in 3 changes of PBS, and colored for 15 min with 0.1% aqueous solution of crystal violet (CV). The wells were rinsed extensively in several changes of PBS, the dye was solubilised with either a mixture of ethanol and acetone in proportion 4:1 for the Gram-positive strains, or 70% ethanol for the Gram-negative strains. The absorbance of the solubilised dye was measured at 570 nm.

Results and Discussion

Culture media may have different effect on bacterial growth and biofilm formation

Preliminary experiments on the growth dynamics of the tested strains inoculated on 96-well polystyrene plates have confirmed that on hour 24 of culture all strains in all media are in the stationary phase (data not shown).

For the purpose of the present study we measured the OD 620 of the wells as an indicator of the bacterial growth and then carried out the procedure for CV staining to measure biofilm in these same wells. On Fig. 1. is shown the comparison between non-biofilm and biofilm growth within the same experiments.

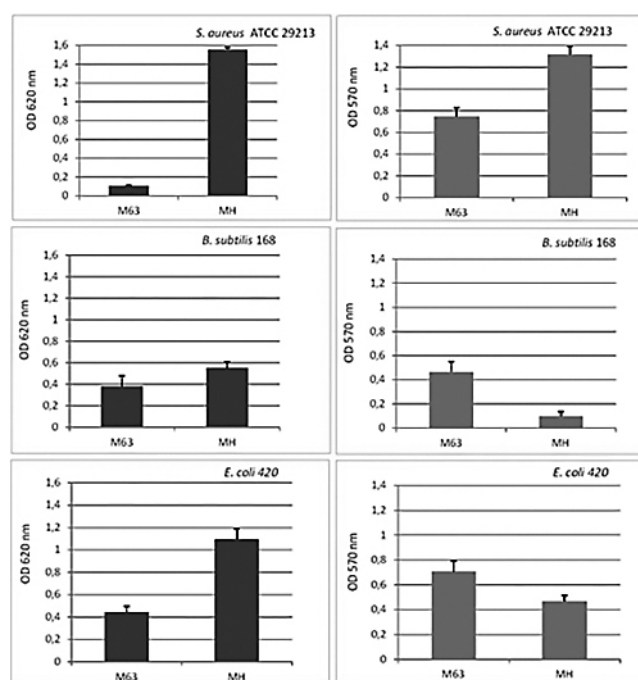


Fig. 1. Comparison of the effect of growth medium on bacterial growth (dark bars, absorbance at 620 nm) and biofilm formation (light bars, absorbance at 570 nm). The experiment was performed in 96-well plates, after cultivation for 24 hours at 37°C.

The results show that conditions that do not stimulate growth in broth may be promoting biofilm growth, and vice-versa. Thus *S. aureus* ATCC 29213 does not grow well in the liquid phase of M63 medium however it develops significant amount of biofilm in these same wells. While the MH broth culture promotes the planktonic growth of *B. subtilis* 168 compared to M63 medium, this strain produces more biofilm in the minimum salt medium than in MH. Likely is the situation with *E. coli* 420.

The development of biofilms in nutrient-limited media such as M63 has rarely been compared to that in rich media. Strain specificity of nutrient demands has been reported for *Campylobacter jejuni*

(Teh et al., 2016). The impact of the nutritional stress may be observed not only as changes of biofilm biomass. Thus, for *Burkholderia pseudomalei* it has been shown that growth of both plankton and biofilm under nutritional stress promotes the tolerance to antibiotics (Anutrakunchai et al., 2015, 2018). These results imply that the screening for anti-biofilm substances may benefit from parallel experiments performed under different nutritional conditions.

Culture media may have diverse effects on the dynamics of biofilm growth

The processes of bacterial biofilm development follow the same general pattern (Souza dos Santos et al., 2018). Initially, the motile bacteria approach the surface that is to be colonised and explore it by moving to and fro. This has been observed also in real time in our experiments by the application of TIRM to *E. coli* 420 (Velinov et al., 2011). This is the phase of the reversible adhesion. From one moment on some bacteria lose their flagella and attach irreversibly to the substratum. This is followed by attached growth of the bacteria starting with the formation of microcolonies, accumulation of biofilm biomass and, finally, mobilization of some cells and their detachment from the biofilm in search of novel niches. The latter process is little known, expectedly it is related with processes of degradation or loosening of the biofilm matrix (Huang et al., 2018).

While there is general agreement that biofilm development follows the above-outlined stages, it has to be taken into account that these processes exhibit complex spatio-temporal dynamics (Lee et al., 2019). The present experimental results show that the time course of biofilm growth may vary dependent on the growth medium. Fig. 2 shows the comparison between the time-courses of the biofilm development by *P. aeruginosa* PAO1 in three media, followed diurnally, on days 1, 2, and 3. It is clearly seen that in M63 medium the biofilm biomass increases between hours 24 and 72. Just the opposite, in LB the biofilm reaches its maximum value on hour 24 and this is followed by diminution of the biomass. Most probably, in the rich medium, once the biofilm has reached the state of significant growth, the bacteria sense some stimuli to become mobilized, leave the sessile community and explore new niches. This dynamics has to be considered when choosing the appropriate protocols for either inhibition of biofilm growth, or dispersal of pre-formed biofilms. And, finally, the data show that MH is promoting less than the other two media the

sessile growth of this strain. In this medium, the biofilm reaches maximum development on day 1, followed by some diminution at later intervals.

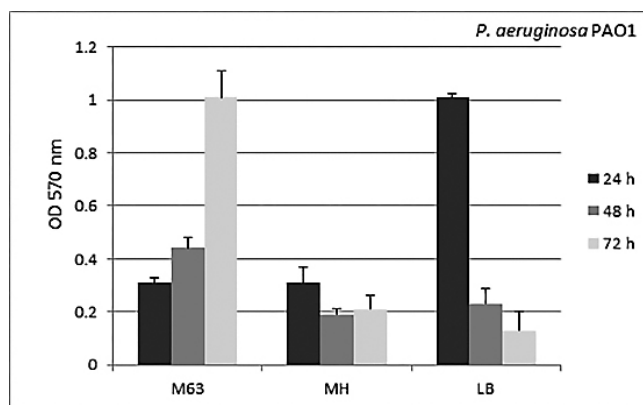


Fig. 2. Time-course of biofilm development by *P. aeruginosa* PAO1 in three growth media.

These time-course responses of *P. aeruginosa* PAO1 to the growth media characterize this strain but should not be extrapolated to other strains of this species. Thus, a vast variety of nutritional preferences between the three growth media have earlier been demonstrated for a big collection of clinical and environmental isolates of *P. aeruginosa* (Cullen et al., 2015).

The spatio-temporal dynamics of biofilm development and especially the mobilization and detachment of bacteria at later stages of the sessile-growth process have another important aspect: cells detached from biofilm may exert significant differences from cells grown as plankton. Thus, detached cells of *S. aureus* differed from plankton in their physico-chemical characteristics, cytotoxicity to He-La cells and production of virulence factors (Khelissa et al., 2017). Detached cells of *P. aeruginosa* had higher susceptibility to benzakonium chloride than cells grown as plankton (Khelissa et al., 2019). Therefore, future experiments aiming to find substances applicable for biofilm control should include also the effects on biofilms at the advanced stage of development, the detachment phase.

Strain-specific responses of biofilm growth to temperature

The set of *E. coli* K-12 strains used in the present study have been often included in experiments in our laboratory, with variable results between M63 and MH media (Vacheva et al., 2011, 2012 a, b) These studies have shown strain-specific responses to the growth medium.

In the present study we show that, in the same medium - MH, the biofilm-formation response to

growth temperature may also be strain-specific (Fig. 3). The duplicate set of experiments started from the same overnight inocula for each strain, diluted 1:100 in MH, and two identical plates were prepared and incubated at either 20° or 37°C. As expected, the OD 620 nm showed that in the liquid phase all strains grew better at 37°C (Fig 3A). But the biofilm developed in these same wells did not show such a strict pattern.

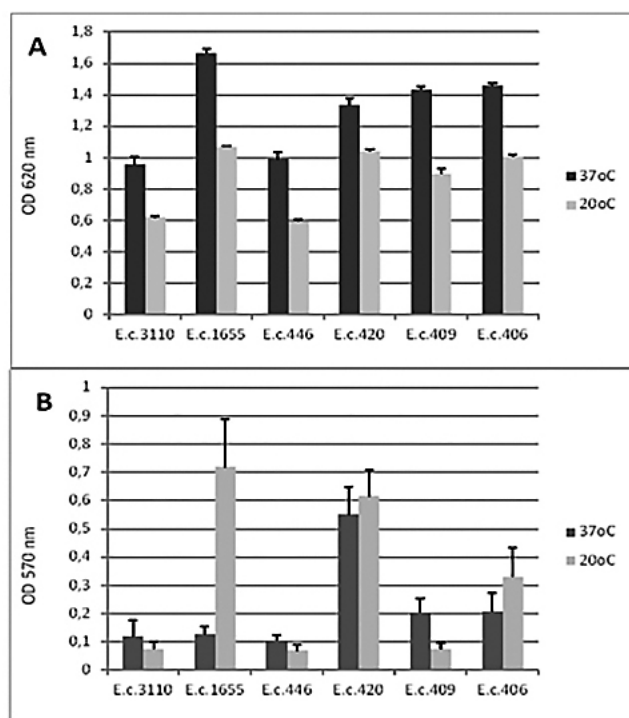


Fig. 3. Effect of cultivation temperature on the growth (A) and biofilm formation (B) of *E. coli* K-12 strains in MH broth.

Strains 3110, 409 and 446 have previously been shown to form almost no biofilm at 20°C in M63 medium (Vacheva *et al.*, 2012b). The present experiments performed in the MH medium confirmed this peculiarity for 3110 and 446. However at 37°C an increase of biofilm biomass was registered for strain 409 (Fig. 3B).

For their biofilm formation, strains 1655, 420 and 406 demonstrated preference for 20°C (Fig. 3B). Such a temperature preference has been shown earlier for many other K-12 strains though strain specific responses have also been observed (Mathlouthi *et al.*, 2018). Investigations on other bacterial species have also focused on the effect of temperature on biofilm. Temperatures lower than 35°C promoted sessile growth of *Salmonella enterica* serovar Enteritidis (Iliadis *et al.*, 2018) and *Acinetobacter baumannii* (De Silva *et al.*, 2018). Cultivation at 37°C of *Streptococcus uberis* resulted in bigger biofilm biomass than at lower

temperatures. Different data for the temperature preferences of *Listeria monocytogenes* have been reported by different laboratories (Nowak *et al.*, 2015; Dhowlaghar *et al.*, 2018). Growth temperature is an important factor not only because it affects biomass. It has been reported that growth temperature of *S. aureus* and *P. aeruginosa* influenced biofilm resistance for disinfectants (Lee *et al.*, 2015; Abdallah *et al.*, 2015a, b).

Summing up the present experimental data, it can be concluded that the species- and strain-peculiar responses to growth conditions have to be considered when preparing experimental designs directed to the identification of novel substances with putative application for biofilm control of the model Gram-positive and Gram-negative strains.

Acknowledgements: This study was part of the Bilateral Project between the Stephan Angeloff Institute of Microbiology, Bulgarian Academy of Sciences, Sofia, Bulgaria and National Research Center, Cairo, Egypt – 2019 "Suppression and destruction of biofilm formation using novel antibiofilm nanostructure materials: A multi-disciplinary approach". Dr. Ts. Paunova-Krasteva acknowledges the financial support of the Bulgarian Ministry of Education and Science under the National Research Programme "Young scientists and postdoctoral students" approved by PMC № 577/17.08.2018

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Influence of Virus Diseases on the Yield of the Medicinal Plant *Echinacea purpurea*

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Abstract

Symptoms of virus diseases on the leaves of the medicinal plant species *Echinacea purpurea* (L.) Moench. – purple coneflower were observed in the trial fields of the Institute of Rose, Essential and Medicinal Cultures (IREMC) near Kazanlak and private gardens in Kazanlak, Bulgaria. Virus diseases, caused by the following viruses were established on *E. purpurea*: *Alfalfa mosaic virus* (AMV), *Cucumber mosaic virus* (CMV), *Tobacco mosaic virus* (TMV), *Tomato spotted wilt virus* (TSWV) and *Potato virus Y* (PVY). These viral pathogens existed in *Echinacea* plants in individual or most commonly in mixed infections. The symptoms caused by viral pathogens were spotting (chlorotic spots of different shape and size) on the leaves, sometimes typical mosaic, reduction in leaf size and flower head diameter and especially decrease in the height of the stems, called dwarfing. The results of the study have shown, that the most widely spread were CMV, AMV, TMV - over 45 % and TSWV – nearly 30 %. Clear differences were detected in the yields of roots, seeds and leaves between Group I of symptomless *Echinacea* plants (practically healthy without symptoms or damage) and the spotted *Echinacea* plants (Group II – of spotted and dead plants and Group III - of spotted, but vital plants, in which the viruses were established. The spotted *Echinacea* plants were shorter (dwarfed) in comparison with the symptomless plants with fewer leaves and flower heads. The yield of the seeds, leaves and roots of the spotted plants was twice or several times lower in comparison with the symptomless plants

Keywords: influence on the yield of *Echinacea purpurea* plants, caused by AMV, CMV, TMV, TSWV.

Резюме

Симптомите на вирусни болести по листата на медицинското растение *Echinacea purpurea* (L.) Moench. бяха наблюдавани в Института по Розата, етеричномаслените и медицински култури (ИРЕМК) в Казанлък и частни градини около Казанлък, България. Вирусни болести, причинени от следните вируси: *Alfalfa mosaic virus* (AMV), *Cucumber mosaic virus* (CMV), *Tobacco mosaic virus* (TMV), *Tomato spotted wilt virus* (TSWV) и *Potato virus Y* (PVY) бяха установени на *E. purpurea*. Вирусните патогени заразяват ехинацеевите растения по-често като смесена инфекция от няколко вируса и по-рядко единична инфекция от един вирус. Вирусните патогени причиняват симптоми на напетняване (хлоротични петна с различна форма и големина), понякога типична мозайка, намаляване размера на листните петури и диаметъра на съцветията и специално намаляване височината на стъблата, наречено вджуджаване. Резултатите от проучването показват, че най-широко разпространени са CMV, AMV и TMV – над 45 % в насаждение и TSWV – почти 30 %. Отчетливи разлики в добивите на корени, листа и семена от ехинацееви растения бяха доказани в разпределените по групи растения: Първа група – безсимптомни (практически здрави растения - без вируси или с латентна инфекция, без проява на симптоми), Втора група – напетнени от вирусни патогени растения - изсъхващи и загиващи и Трета група – напетнени от вирусни патогени растения, но жизнеспособни. Напетнените ехинацееви растения бяха с намален хабитус (вджуджени) и с намален брой листа и съцветия в сравнение с безсимптомните. Добивът на семена, корени и листа при напетнените растения беше от два до няколко пъти по-нисък в сравнение с безсимптомните ехинацееви растения.

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Introduction

Virus diseases reduce yields and deteriorated the quality of the agricultural crops. The results of the research showed that the economically important purple coneflower (*Echinacea purpurea*) viruses in Bulgaria are *Alfalfa mosaic virus* (AMV), *Cucumber mosaic virus* (CMV), *Tobacco mosaic virus* (TMV) *Tomato spotted wilt virus* (TSWV) (Dikova, 2011; 2012; 2016; Dikova *et al.*, 2010; 2013). In Ukraine, it was found that the purple coneflower plants were infected with TSWV – in 2012, PVY – in 2013, and TRV – in 2014 (Dunich and Mishchenko, 2015; Mishchenko *et al.*, 2015). CMV, PVY, TMV и TSWV in a mixed infection in only one purple coneflower plant were established by Horvath *et al.* (2006) in Hungary. The most harmful is CMV, which has circulated on the *E. purpurea* plants for several years, because *Echinacea* plants are cultivated for harvesting as a perennial crop.

E. purpurea (L.) Moench.), the most powerful immunostimulating agent among the medicinal plants is widespread in natural habitats in the North of the USA and southern Canada. The soil and climatic conditions in Bulgaria are very suitable for cultivation of this medicinal plant species. Apart from the reduced quantities of the root, leaf and seed yields, *Echinacea* plants affected by viral pathogens have pathogen-induced changes in the chemical composition most probably reflected on the immunostimulating properties of this medicinal crop. According to Bellardi *et al.*, (2001) the concentration of Germacrene D and of some alkylamide components in root extracts of *Echinacea* plants, infected by CMV is significantly decreased in comparison with the root extracts of healthy plants.

The objective of the research was to establish the influence of virus diseases on the yield of the medicinal plant species *E. purpurea* (L.) Moench.

Material and Methods

E. purpurea plants were analyzed in the Department “Plant Protection” in the Institute of Soil Science, Agrotechnologies and Plant Protection (ISSAPP) “Nikola Poushkarov”, Sofia, Bulgaria. The methods of analysis were ELISA (DAS-ELISA) (Clark *et al.*, 1977) and the indicator method using indicator test plants (Kovachevsky *et al.*, 1985). Samples of *Echinacea* plants were collected from the trial fields of the Institute of Rose, Essential and Medicinal Cultures (IREMC), near Kazanlak, Bulgaria and from private gardens

in Kazanlak. Some of the *Echinacea* plants were with symptoms of virus diseases while others were symptomless. Each sample from an individual *Echinacea* plant was analyzed with DAS-ELISA kits, purchased from LOEWE, Biochemica (Germany) for the following viruses: *A. mosaic virus* (AMV), *C. mosaic virus* (CMV), *T. mosaic virus* (TMV), *T. spotted wilt virus* (TSWV) and *Potato virus Y* (PVY). DAS-ELISA tests were carried out according to detailed descriptions in other publications (Dikova, 2012; 2016). The indicator method using differential indicator (test) plants, such as *Nicotiana tabacum* cv. Samsun NN, which was a differential indicator for TSWV, ToMV and CMV is very suitable for visual detection of each of these plant viruses (Dikova, 2016; 2019). In addition to DAS-ELISA some viruses, such as TSWV, ToMV and CMV were also identified by the reaction of the indicator (test) plants: *Nicotiana tabacum*, cv. Samsun NN.

The negative influence of the disease-causing viral pathogens, affecting the quantity and quality of the yield of roots, leaves and seeds from these plants was studied in a three- year *Echinacea* plantation in a private garden near IREMC, Kazanlak. The data for each of these 7 *Echinacea* plants were presented in another paper (Dikova *et al.*, 2013). The present study recapitulates the initial data on the following groups of plants: Group I included two symptomless *Echinacea* plants; Group II were three spotted and perishing plants and Group III were two spotted, but vital *Echinacea* plants without drying.

Results and Discussion

Symptoms of virus diseases were often observed in private gardens, adjacent to vegetable gardens and viral pathogens were afterwards established by DAS-ELISA and the indicator method. The viral pathogens caused damage to the *Echinacea* plants such as stunted plant habitus (mainly height and width of the stems), decrease in leaf laminae and the diameter of the flower heads. Typical symptoms caused by CMV and TSWV viruses on purple coneflower plants originally were spotting, mosaic symptoms (Fig. 1) and dwarfing – decreasing the habitus of the plants. The difference between the symptomless *Echinacea* plants (Fig. 2) and the spotted virus - infected plants (Fig. 3) can be determined by some of these biometrical indices. TSWV caused typical symptoms as like light yellow to bright yellow circular or irregular spots on the middle and lower stages of leaves that subsequently turned into necrotic spots.



Fig. 1. *E. purpurea* leaves with symptoms of mosaic, caused by CMV.



Fig. 2. Symptomless *E. purpurea* plant



Fig. 3. Tuft of *E. purpurea* plants -spotted and dwarfed plants by viral pathogens



Fig. 4. Root sprout, used for propagation and preparation of seedlings



Fig. 5. *E. purpurea* seedlings-spotted by viral pathogens



Fig. 6. *E. purpurea* plant tuft with symptoms of spotted and dried leaves by artificial CMV/ TSWV infection

The mixed infection of several viral pathogens caused dwarfing of some plants (Fig.3) and as a consequence decreased quantity and quality of leaves (herba) and flowers. Symptoms (typical mosaic symptoms) of single infection with *Cucumber mosaic virus* (CMV) on the *Echinacea* leaves are shown on Fig. 1. The purple coneflower plants are often propagated by root sprouts (Fig. 4) and then the incidence of the virus diseases in the adult plants is certain (Fig. 5).

The results on the viruses established in *E. purpurea* plants by DAS – ELISA are presented in Table 1.

CMV was established by DAS-ELISA as well as by the reaction of the indicator (test) plant *N. tabacum* cv. Samsun NN (Fig. 7) and TSWV

was proven by the reaction of the *N. tabacum* cv. Samsun NN too (Fig. 8).

The yield of seeds, obtained from the symptomless *E. purpurea* plants (Group I) was over 20 times higher in comparison with the yields of Groups II and III – plants with symptoms of virus diseases (Table 2). Group I were practically healthy without symptoms and without viruses *Echinacea* plants, or viral pathogens were in very low concentrations and caused latent infection. The yield of roots in Group I – the symptomless (practically healthy) *Echinacea* plants was 11 times higher in comparison with the yield in Group II of spotted and dead plants and 4 times higher in comparison with the yield of Group III of spotted, but vital plants (Table 2). The yield of leaves (herba) from

Table 1. Data about economically important viral pathogens established on *E. purpurea* by DAS-ELISA

Name of viral pathogen	Number of analyzed plants	Number of plants with viruses	% diseased plants in comparison with all tested plants
<i>Cucumber mosaic virus</i> (CMV)	53	24	45.5
<i>Alfalfa mosaic virus</i> (AMV)	36	20	55.5
<i>Tomato mosaic virus</i> (TMV)	12	6	50.0
<i>Tomato spotted wilt virus</i> (TSWV)	11	3	27.3
<i>Potato virus Y</i> (PVY)	32	1	3.13



Fig. 7. Systemic symptoms of CMV on differential test plant *N. tabacum* cv. Samsun NN



Fig. 8. Systemic symptoms of TSWV on differential test plant *N. tabacum* cv. Samsun NN

Table 2. Comparison between the yield of roots, leaves and seeds from symptomless (practically healthy) *E. purpurea* group of plants and virus-spotted *E. purpurea* groups of plants, and biometrical indices influenced by virus diseases

Yield	Yield in g per groups of <i>Echinacea</i> plants			Average yield in g per groups of <i>Echinacea</i> plants		
	Group I – symptomless (practically healthy) plants	Group II – spotted by viruses perishing plants	Group III – spotted by viruses vital plants	Group I – symptomless (practically healthy) plants	Group II – spotted by viruses perishing plants	Group III – spotted by viruses vital plants
Seeds in group	42	3	1	21	1	0.5
Leaves (herba) in group	875	0	195	438	0	98
Roots in group	1565	212	355	783	71	178
Stem height in cm	164	167	125	82	56	63
Number of rosette leaves	39	24	19	20	8	10
Number of stem leaves	390	124	77	195	41	39
Number of flower heads	70	32	32	35	11	16

dead and perishing *Echinacea* plants was zero, and the yield of spotted and vital plants was fourfold lower in comparison with the symptomless plants (Table 2). The biometrical indices in Group I of symptomless plants were higher in comparison with the biometrical indices of the plants in the groups with symptoms of virus diseases. The stem height of Group I of symptomless plants was 26 cm taller in comparison with Group II of spotted and dead plants and 19 cm taller than in Group III of spotted, but vital plants (Table 2). The number of the stem leaves in Group I of symptomless plants was 4 times higher in comparison with Group II of spotted and dead plants and 5 times higher in comparison with Group III of spotted, but vital plants (Table 2).

The flower yield (flower heads) in Group I of symptomless plants was 2,9 times higher in comparison with Group II of spotted and dead plants and twice higher in comparison with Group III of spotted, but vital plants (Table 2).

Wilting and drying, caused by *C. mosaic virus* (CMV) of adult vegetable plants were proven on the field and in greenhouses (Kovachevsky *et al.*, 1985). Perishing *Echinacea* plants with mosaic symptoms, caused by CMV on the leaves were established in *Echinacea* tufts too (Dikova *et al.*, 2013).

The means of virus control in *Echinacea purpurea* crops are preventions of viral invasion by insect pest control of transmitters: aphids for AMV,

CMV and PVY and of thrips for TSWV after investigations of the plantations. TMV control includes phyto-sanitary means against this mechanically transmitted virus.

Conclusion

The results from the studies of the symptoms on virus diseases, caused by CMV, AMV, TSWV, TMV and PVY on the yield showed that the viral pathogens caused serious injuries on the *E. purpurea* plants that has worsened their yield of roots, seeds and leaves in terms of quantity. ELISA method (DAS-ELISA) permitted the analysis of a large number of *E. purpurea* samples for several viral pathogens simultaneously. The indicator method using a differential indicator (test) plant, such as *N. tabacum* cv. Samsun NN, which was a differential indicator for TSWV, TMV and CMV is very suitable for visual detection of each of these plant viruses (Dikova, 2016).

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Events

72 Years since the Establishment of the “Stephan Angeloff” Institute of Microbiology at the Bulgarian Academy of Sciences

CONGRATULATORY ADDRESS BY THE DIRECTOR OF THE INSTITUTE

Honoured guests, dear colleagues, ladies and gentlemen,

Today, it is with pride, self-esteem and confidence that we are celebrating the 72nd anniversary of the Institute of Microbiology “Stefan Angeloff” at the Bulgarian Academy of Sciences!

Later this year we all shall also celebrate the 150th anniversary since the founding of the Bulgarian Academy of Sciences – a symbol of our nation and one of the pillars of our national identity and heritage. Founded in the distant 1869 as Bulgarian Literary Society, it was later (in 1911) renamed Bulgarian Academy of Sciences. Both praised and condemned in the years, the Academy has been able to assert itself not only as a scientific but also as one of the main enlightenment and spiritual centres in the country. It has always been a strong proponent of the free development of science and technology, and to this day continues to keep up the spirit and scientific thought and significantly contribute to the development of Bulgarian culture, economy and the state as a whole. As a leading national centre of science, the Academy affirms the prestige of our nation and promotes the image of Bulgaria within the united European family as well as around the world. “It is up to us to be equal to the other European nations”, said Vasil Levski, the Apostle of Freedom, and this covenant is kept and passed on to the next generations of scholars and researchers, filling our everyday lives with substance and meaning!

Today's formal assembly is a worthy opportunity for us to state with openness and conviction: our Institute is a recognized centre of microbiological sciences in Bulgaria and Southeast Europe, the Institute has gained European popularity and identity, the Institute has educated and trained a multitude of microbiologists of international renown, and, last but not least, it is an attractive training centre for young professionals in microbiology, immunology and biotechnology.

Founded on March 7, 1947 by the internationally acclaimed Bulgarian scientist Academician Stefan Angeloff, the Institute of Microbiology at the Bulgarian Academy of Sciences has been consistently developing the main branches of the microbiological science - bacteriology, virology, mycology, general and industrial microbiology, and immunology. The founder, whose name the Institute has been proudly bearing since 1997, was also the person to lay the foundations of microbiology as a science in Bulgaria. He introduced the diagnostics of syphilis, cholera, and epidemic typhus, as well as of swine fever, foot-and-mouth disease, dourine of horses, production of anthrax vaccine and serum, malein and tuberculin, and supervised the fight against tuberculosis and cholera. In 1913, Stefan Angeloff developed an original vaccine against cattle plague, whose production was organized in the village of Ladzhakoyi (Bourgas Region), and which for the first time curbed the spread of this devastating epizootic in Europe. He was one of the founders and repeatedly elected dean of the Faculty of Veterinary Medicine at Sofia University, and from July 14, 1947, having taken over the leadership of the Institute of Microbiology at the Bulgarian Academy of Sciences, until 1961 dedicated himself to the development and consolidation of the Institute and his own scientific school of thought. For his outstanding merits, Academician Angeloff was conferred with high national and international honours: Doctor Honoris Causa of Berlin University (1932) and of the Faculty of Veterinary Medicine in Hanover (1940), Honorary Member of the Imperial Leopoldina Carolina German Academy of

Natural Sciences in Halle (1940), Corresponding Member of the Academy of Agricultural Sciences (Berlin) (1955). Professor Angeloff was Rector of Sofia University "St. Kliment Ohridski" (1941-42), was granted the honorific titles Corresponding Member (1943) and Academician (1947), and twice Dimitrov Prize Laureate for his overall scholarly work (1950) and for Bistrin preparation (1952).

Today, the Institute as our legacy is a well-established national research centre in the field of microbiological sciences. The strategy of its activities is focused on issues of major importance to the healthcare, food and pharmaceutical industries and national ecology. In addition, a substantial share of its activities is taken up by teaching and training graduate and doctoral students. Our scholars are sought-after experts in many international and national boards and committees of ministries, agencies and organizations.

Contemporary scientific research at the Institute of Microbiology encompasses a number of major sections of microbiology.

In the area of infectious bacteriology, factors and mechanisms of bacterial virulence are intensively studied: lipopolysaccharides, membrane proteins, enzymes; pathogenesis and ecology of yersiniosis, colli-bacteriosis, salmonellosis and other food zoonoses, molecular epidemiology of tuberculosis; genetically engineered bacterial vectors to create a new generation of vaccines for classical swine fever and other diseases; testing of various natural and synthetic products for antibacterial activity; bacterial resistance to antimicrobial agents; vaginal lactobacilli – molecular and genetic analysis and their role in sexually transmitted infections; bacteriocins; development of methods for microbiological control of food products.

In the field of virology, experimental chemotherapy of viral infections is also being researched; development of effective anti-adenoviral chemotherapy; antivirals against toga- and flaviviruses; new viral inhibitors of synthetic and natural origin (plant, microbial); mechanisms of action of viral inhibitors; basis of resistance to viral inhibitors; biological response modifiers - immunomodulators, interferon inducers, antioxidants; combined effects of viral replication inhibitors, antiviral microbicides - a screening system, development of a new generation of antiviral disinfectants; viruses in the etiopathogenesis of Balkan endemic nephropathy; viruses - diabetes; human papillomaviruses - cervical cancer; influenza viruses - influenza infection pathogenesis, use of antivirals against influenza.

In infectious immunology, immunopharmacological tests are carried out on substances of natural origin (microbial and plant) and synthetic products; their immunomodulatory effect is tested in experimental models of infections and tumours in laboratory animals; autoimmune disorders caused by infection; a concept of physiological autoimmunity has been developed; mechanisms of autoimmunity control; investigation into the role of natural autoantibodies in autoimmune diseases; restoring tolerance to DNA with genetically engineered chimeric molecules as a new approach to the treatment of systemic lupus erythematosus.

In the field of applied microbiology, a large-scale genetic analysis is underway of microorganisms used in the food and pharmaceutical industries - lactobacilli, streptomycetes, yeasts. Microbial cell response to physiological stress is characterized; selection and properties of microorganisms used as starter cultures in the production of food products; work on the production, properties and spectrum of application of biologically active microbial products - enzymes (including thermostable), such as superoxide dismutase, polyonase, inulinase, proteases, pectinases, amylases, TAQ-polymerase, neuraminidase; organic acids, vitamins and growth factors; amino acids; biosurfactants; antibiotics; cell cultures from endemic plants as a source of biologically active substances for the food and pharmaceutical industries; mathematical modelling of biotechnological processes; microbial ecology - microbiological monitoring of soils and waters; the role of microorganisms in bioremediation and biodegradation; biogas technology.

The overall research activity of the scientists in the above areas was highly evaluated in the International audit by experts of the European Science Foundation and the European Federation of Academies of Sciences in 2009. The Institute was given maximum assessment scores and was placed among the top Academic Institutes. The 2017 evaluation of the Ministry of Education and Science also ranked the Institute among the elite institutes. The prestigious valuation, which was mainly the result of the consistent and constant efforts of the scientists at the Institute over the last 10 years, as well as of the national and international prestige of the team and leadership, is certainly motivating and requires a large amount of work for ever

better achievements.

One of the most significant events in the Institute's life was its admission to the family of institutes associated with the Pasteur Institute (Paris) on June 26, 2004. We are one of the six European institutes with such status, along with the Pasteur Institutes of Brussels, Saint Petersburg, Lille, Athens and Sapienza (Rome). It is a recognition of great significance for the overall work of our Institute. In addition, it should be pointed out that the Institute is the founder and headquarters of the Balkan Society of Microbiology, as well as a coordinator and centre of cooperation in Southeast Europe

At present, the Institute has 5 departments, 4 sections and 14 laboratories and an individual membership of 143, of which one academician, two corresponding members, one professor, 6 doctors of science, 19 associate professors, 28 senior assistants and 27 assistants. It is noteworthy that approximately 20% of the research staff are young people under the age of 35, which renders our institute one of the most creditable and promising in the Academy and the country. Moreover, our young colleagues are traditional winners of the awards of the Akad. Prof. Dr. Stefan Angeloff Foundation for excellence in the field of microbiology, the Prof. Marin Drinov Award for young scientists under 35 for achievements in the field of biological sciences, the Pythagoras Prize for young scientists in the field of plant biotechnology as well as the Pythagoras Prize for established scientists in the field of biomedical sciences. In the past year, scientists from the institute have also won the Science Award for Excellence of the Union of Scientists in Bulgaria, a Diploma of Excellence from the Bulgarian Academy of Sciences, the government prize for the most cited scientist in the field of engineering and environment for 2017/2018, an honorary plaque for contribution to the development of science at the University of Food Technologies and others.

In the context of a funding gap, the Institute's researchers tirelessly and committedly work on projects under the European Union and European Space Agency programs, the National Science Programs, the Pasteur Institutes Network, National Fund for Scientific Research (NFNI), Bilateral Research Programmes (EBR), etc. in cooperation with foreign partners, organizations and companies. Work is also being actively initiated at the competence centres, opening a five-year horizon for development. Projects implemented with teams from the Bulgarian Academy of Sciences, universities, Bulgarian companies, also have a fair share in overcoming the financial shortages of the Institute.

By formally celebrating this important event, today we pay tribute and respect to our predecessors, who have worked actively and consistently to promote the Institute's position and achievements. In the years of political transition, filled with many hardships and vicissitudes, we should sincerely thank Academician Angel Simeonov Galabov, Director of the Institute for 17 years, for his incessant aspirations to raise the name and prestige of the Institute, the values of the scientific community in the Academy and defend its interests and ambitions. We wish you strong health and creative longevity, Academician Galabov!

The history of the Institute will continue to be enriched with names, facts, events and analyses, and completed with the contemporary scientific, research and development work of our scientists, however, let us not forget the lessons of the past and appreciate the contribution of our celebrated predecessors, who outlined the future of the Institute as an integrated and unique centre for research and innovation in microbiology, with a deserved place in the European science domain.

Congratulations on the 72nd anniversary of our Institute, dear colleagues and guests!

Cor.-Mem. Hristo Naidenski, D.V.M.
Director of the Institute





Cronicle

5th National Congress of Virology Days of Virology in Bulgaria with international participation 2-4 October 2019, Sofia, Bulgaria

Dear colleagues,

On behalf of the Organizing Committee, I would like to invite you to participate in the 5th National Congress of Virology/Days of Virology in Bulgaria which is held in Sofia, at October 2nd-4th, 2019.

All branches of the modern virology will be considered during the congress: general (fundamental) virology, medical virology, veterinary virology and plant virology.

The program will includes plenary lectures done by invited speakers (lasting 30 minutes), oral (15-minutes) reports and poster presentations.

We shall be happy to hear your recent research achievements in the mentioned above domains of the virology.

The registration details will be given in the website: <http://www.daysofvirology2019.com/>

Professor Angel S. Galabov, MD, DSc

Regular member of the Bulgarian Academy of Sciences
President of the Congress Organizing Committee

Acta Microbiologica Bulgarica

GUIDE FOR AUTHORS

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

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

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

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

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**The edition of *Acta Microbiologica Bulgarica*
volume 35 issue 2 is financially supported
by
The National Science Fund of Bulgaria
(KP 06 NP-13/2018)**