PHYLOGENETIC ANALYSIS OF COAT PROTEIN GENE OF TOMATO MOSAIC VIRUS ISOLATES CIRCULATING IN UKRAINE

Tomato mosaic virus (ToMV) induces highly infectious disease of vegetables, whereas use of virus-contaminated seed may lead to complete yield loss. This work was aimed at studying phylogenetic relationships of Ukrainian tomato isolates of ToMV with its known isolates by comparing nucleotide sequence of coat protein gene. ELISA, TEM, RT-PCR, sequence analysis using MEGA 5 software, and statistical methods. cDNAs of two novel Ukrainian isolates ToMV-ukr-5 and ToMV-ukr-10 corresponding to coat protein (CP) gene were sequenced and compared with other published ToMV sequences. On the constructed phylogenetic tree, ToMV isolates were grouped into two separate clusters. In addition to novel Ukrainian isolates ToMV-ukr-5 and ToMV-ukr-10, the first and larger cluster contained nearly all virus isolates used in this study with high (96-98.9 %) level of homology to Ukrainian isolates. The larger cluster was clearly separated into two subclusters: one grouping isolates with over 98.7 % identity with Ukrainian isolates and the other containing three strains and isolates with 96.1 % identity (tomato isolate SL-1, strain camellia isolated from a decorative plant, and isolate Dahlemense DSMZ PV-0135). Two novel Ukrainian isolates ToMV-ukr-5 and ToMV-ukr-10 have been isolated from tomato plants cultivated in open field conditions in different regions of Ukraine. Phylogenetic analysis confirmed high identity of Ukrainian isolates between themselves and with other published ToMV sequences. Ukrainian isolates were most homologous (>98 %) to Brazilian isolate Hemoeracallis, to Chinese isolate G2, and to the following tomato isolates: AH4, Queensland, ToMV-tom and LS-K, S14, and FERA_160205. The high level of homology was traced independently of the source of virus isolation, its plant host and their geography.

Key words: Tomato mosaic virus, tomato plants, phylogenetic analysis, isolates, sequencing, Ukrainian ToMV isolates.

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ISOLATION OF BACTERIOPHAGES WITH LYTIC ACTIVITY AGAINST A NEWLY IDENTIFIED PANTOEA AGGLOMERANS

In a consequence of agricultural human activity, a set of phytopathogenic bacteria gain new properties and ability to cause diseases in animal and human organisms. Moreover, bacterial loss of sensitivity to antibiotics becomes more increasing threat. The most effective alternative method of processing of plants are bacteriophages. The aim of this work is isolation and identification of a vegetable enterobacteria and search of its specific bacteriophages. Methods: biochemical identification of bacteria, analysis on sensitivity to antibiotics by means of disks, titration and accumulation of virus, electronic microscopy. Results: from onions samples with symptoms of a bacteriosis several bacteria were isolated. One of them was identified as Pantoaea agglomerans. The sensitivity of this isolate to antibiotics was investigated, the resistance to cefalexin and norfloxacin is revealed. The bacteriophage of this isolate was isolated from waste waters. The morphology of a bacteriophage is investigated by means of electronic microscopy, the virus belongs to the Myoviridae family. Phytopathogenic properties of bacteria and the antibacterial activity of phage isolate were investigated on potatoes in vitro. P. agglomerans led to development of a bacteriosis on potatoes cubes, and the isolated bacteriophage successfully inhibited its growth. Conclusions: This study demonstrated that common vegetables such as onions could be a source of human pathogenic bacteria. In this work, we isolated P. agglomerans, member of family Enterobacteriaceae. Taking into account that this bacteria was unsensitive to some antibiotics, it can be regarded as an alarming sign. The use of bacteriophages could solve problems of antimicrobial resistance and protecting of crops from bacterial infections. Isolated bacteriophage from waste waters inhibited growth of P. agglomerans in vitro showing that it could be considered as a part of phage drugs.

Key words: Pantoaea agglomerans, onion, phage therapy, bacteriophages.
Materials and methods. The content of brown rot from onions was placed on LB growth medium (gms/l: tryptone – 10, yeast extract – 5, NaCl – 10, agar – 10, pH=7.5). Bacterial cells were stained according to Gram and examined on magnification x100 (MICROMed XS-4130). Type of respiration was established after tests on cytochromoxidase with Hugh-Leifson medium (main solution: peptone – 2, NaCl – 5, KH2PO4 – 0.3, agar – 3, bromothymol blue 0.1% solution – 3 ml, pH=7.1, glucose solution: 10 g glucose, 60 ml dH2O). Biochemical diagnostic features of bacteria were investigated at the clinical diagnostic laboratory of Public national institution "Scientific and practical center of preventive and clinical medicine" of State Directorate for Affairs (PNI "SPC PCM" SDA) with a number of selective medium: Kligler's agar, urease, TTC, Simmons citrate agar and lysine decarboxylase tests. The results of bacterial identification are presented in tab 1. Kligler's test indicated the ability of bacteria to ferment glucose, without H2S release. The urease test was negative, so that the bacteria were not capable to secrete an urease enzyme. TTC test was more positive than negative bacteria showed a spread throughout the medium but after 48h of incubation. Furthermore, the bacteria was able to utilize a citrate in the Simmons citrate agar. The results of lysine decarboxylase test was negative, therefore it indicated the inability of the bacteria to use a lysine as a source of carbon and energy for growth. This isolate was classified as Pantoea agglomerans (formerly Erwinia herbicola) according to the results obtained and to the Bergey's Manual of Systematic Bacteriology (tab. 1).
The results of bacterial susceptibility to antibiotics showed that isolated *P. agglomerans* was resistant to some of them. The resistance to cefalexin, cefotaxime, ceftazidime, ciprofloxacin, chloramphenicol, cefuroxime and norfloxacin was observed that indicates a general resistance to β-lactams. Also, some antibiotics showed boundary values between resistance and sensitivity that indicates development bacterial resistance to them. The results of bacterial susceptibility to antibiotics are presented in the table 2.

### Table 1. Biochemical characteristics of *P. agglomerans*

| Biochemical marker | Kligler’s agar | Urease | TTC (motility) | Simmons citrate agar | Lysine decarboxylase |
|--------------------|----------------|--------|---------------|----------------------|---------------------|
| *P. agglomerans*    | + (glucose fermentation) | -      | +/-           | +                    | -                   |

According to data obtained our isolate of *P. agglomerans* can be the source of antibiotic resistance factors. Taking into account that this bacteria is cross-kingdom pathogen, horizontal gene transfer with plant and human bacteria in the environment can cause an evolving of new multiresistant bacteria.

Then detection of pathogenic properties was conducted on potato cubes. This model system was chosen in order to test bacteria on other typical vegetable as the preliminary phytopathogenicity test on onions yielded the expected positive result. Bacteria caused tissue maceration resulting in rotting and formation of bacterial plaque on slices. *P. agglomerans* isolate showed to be capable of developing bacterial infection in plants and biochemical tests indicate its potential of human pathogen.

According to the obtained results, we attempted isolation of specific bacteriophages from waste waters that is the source of phages of human pathogens due to constant intake of human feces. Waste waters were taken from treatment facilities, then filtrated and plated with *P. agglomerans* by agar overlay method. As a result, specific bacteriophage was isolated (fig.2).

**Figure 2. Phage plaques on bacterial lawn of *P. agglomerans***

Bacteriophage, given work name Eh1, produced small plaques, d<1 mm. After serial propagation, bacteriophages formed negative colonies on bacterial test cultures with no decrease in phage titres, which indicates the lytic cycle of viral reproduction and, moreover, is an indispensable condition for developing phage-based products.

**Figure 2. Phage plaques on bacterial lawn of *P. agglomerans***

Phage isolate Eh1 was investigated with electron microscopy and it was indicated that this virus was member of family *Myoviridae* (fig 3). Isolate had icosahedral head 95X95±4 nm in diameter and tail 120±5 nm in length. Basal plate of the virus was also observed.
To investigate whether the isolate Eh1 is able to suppress bacterial infection in vitro method of potato slices was used again. As a result, bacterial growth on potato cubes was limited by bacteriophage successfully (fig.4).

Slices inoculated only by bacteria displayed the signs of rot while cubes inoculated with bacteria and phage were clear without any symptoms.

In recent years researchers from different parts of the world report more often about isolation of enterobacteria from agricultural plants (5, 8, 17). Plant rhizosphere is high in nutrients and it is enough for human pathogens to live and survive. Violation of crop rotation could be the reason that led organisms such as \textit{P. agglomerans} into new ecological niche. Introduced in agroecosystems with organic wastes, these bacteria adapted and started to invade new organisms – plants. Therefore, using of untreated human excreta contaminated with enteric pathogens to grow vegetables should be strictly controlled (16). Although, successful infection by these bacteria often occurs in co-infection with other phytopathogenic bacteria, this fact do not reduce the risk for human, for example those who eat fresh fruits and vegetables or drink juices. That is why it is important to revise classical strategies of bacterial control and to introduce alternative methods such as bacteriophages.

Conclusions. As the result bacteria was isolated from onion with bacterial rot symptom. Bacteria was identified as \textit{Pantoea agglomerans} due to morphological and biochemical tests. Antimicrobial sensitivity profile of \textit{P. agglomerans} was investigated, isolate showed resistance to some antibiotics. A specific bacteriophage was isolated from waste waters. Phage isolate Eh1 was examined by electron microscopy and identified as a member of family \textit{Myoviridae}.

Ability of the phage to inhibit bacterial infection of \textit{P. agglomerans} in vitro was demonstrated on potato slices.

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ВІДНІЙДА БАКТЕРІОФАГУ З ЛІТИЧНОЮ АКТИВІСТЮ ПРОТИ НОВОЇ ІДЕНТИФІКОВАНІІ ПАНТОЕА АГГЛОМЕРАНС

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THE EFFECTS OF MELATONIN ADMINISTRATION IN DIFFERENT TIMES OF DAY ON THE BROWN ADIPOSE TISSUE IN RATS WITH HIGH-CALORIE DIET-INDUCED OBESITY

The aim of our study was to determine morpho-functional state (area of nucleus, brown adipocytes and also area and number of lipid droplets in each cells, general optical density of tissue) of brown adipose tissue in rats with high-calorie (high fat) diet-induced obesity after melatonin administration in different time of the day (morning and evening). Melatonin was administered daily by gavage for 7 weeks in dose 30 mg/kg either 1 h after lights-on (ZT01) or 1 h before lights-off (ZT11) rats with high-calorie obesity. The effects of melatonin were evaluated on morphometric parameters as well were measured related visceral fat weight and related brown adipose tissue mass. Rats with HCD had huge changes in brown adipocytes morphology, which summarized in become resembles of classical white adipocytes: grown lipid droplets and cells area, but goes down lipid droplets number and optical density of brown adipose tissue. In general brown adipose tissue with above mentioned characteristic from HCD rats lose their ability to conduct strongly thermoproduction function. After melatonin used in rats with HCD arise leveling of pathological changes, which associated with consumption of HCD. Namely, in groups HCD ZT01 and HCD ZT11 we obtain decreased cells and lipid droplets area, increased lipid droplets number and optical density of brown adipose tissue, in relation to group HCD. Therese received changes have evidence about functionally active brown adipose tissue state, which can also dissipate of exceed energy (lipids – triacylglycerols) amount into whole organism during heat production for avoid to its storage in white adipose tissue and in outside adipose tissue. In addition, evening administration of melatonin (group HCD ZT11) demonstrate more activated state of brown adipose tissue and also related visceral weight gain less, than morning (group HCD ZT01). In conclusions, melatonin influence on morpho-functional state brown adipose tissue in rats with HCD, moreover evening administration can use for obesity therapy via its strong action on activate brown adipocytes.

Key words: melatonin, obesity, chronotherapy, adipocytes, high-fat diet, brown adipose tissue.