Part 3. Biosafety

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INTERFERENCE BETWEEN MOSQUITO DENSONUCLEOSIS VIRUS AND CERTAIN ARBOVIRUSES

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Summary. The active ingredient of the preparation Viroden, developed in Ukraine, is the mosquito densovirus. This virus has a wide tissue tropism and affects all phases of ontogenesis. It reproduces itself in the mosquito's salivary gland cells, but unlike arboviruses, it is harmless for humans and vertebrates. It is well established that simultaneous infection of an insect with different viruses is often accompanied by the phenomenon of interference, whereby the reproduction of one or both viruses is suppressed in the insect's body. Consequently, it was reasonable to investigate the results of concurrent infection with an arbovirus and a mosquito densovirus. Laboratory experiments demonstrated that mosquito densovirus suppressed the reproduction of West Nile, Sindbis, and Batai viruses in the mosquito's body, resulting in a significant decrease in their infective titers as well as a reduction in the transmission factor during blood-feeding. The relevance of this research is determined by the increasing levels of biological threats posed by zoonotic transmissible viral infections common to humans and animals. According to the predictions of experts, in light of the processes of globalization and climate change, this may result in the emergence of new pandemics and panzootics

Keywords: mosquito densovirus, West Nile virus, Sindbis virus, Batai virus

Introduction. Densonucleosis viruses (DNVs) are invertebrate-specific viruses belonging to the subfamily Densovirinae within the family Parvoviridae. Densonucleosis is characterized by the hypertrophy of the nuclei in infected cells and cytopathology that leads to either death or loss of vital functions in all stages of infected organisms. Mosquito densoviruses are mosquito-specific, entomopathogenic icosahedral, nonenveloped viruses with a diameter of 20–25 nm. Their single-stranded linear DNA genome ranges from 4 to 6 kilobases and ends in two hairpin structures (Li et al., 2019). All mosquito densoviruses are classified in the genus *Brevidensovirus* (Cotmore et al., 2014).

The mosquito densovirus was first described in a laboratory culture of *Aedes aegypti* (L.) mosquitoes maintained at the Taras Shevchenko National University of Kyiv (Lebedeva et al., 1973). Subsequent studies of its genome revealed that this virus belongs to the Parvoviridae family (Buchatsky and Filenko, 1988; Galev et al., 1989). Subsequently, DNVs were identified in laboratory cultures of mosquitoes belonging to the genus *Aedes* in Thailand (Kittayapong, Baisley and O'Neill, 1999) and in nature in countries such as India (Sivaram et al., 2009), China (Li et al., 2019), and Brazil (Ferreira et al., 2020). Additionally, DNVs have been isolated from C6/36 mosquito cells obtained from *Ae. aegypti* mosquitoes (Jousset et al., 1993; Barreau, Jousset and Cornet, 1994; Boublik et al., 1994; Mossimann et al., 2011; Cataneo et al., 2019). Furthermore, densoviruses have been identified in other mosquito species, including *Culex pipiens* (Jousset, Baquerizo and Bergoin, 2000; Zhai et al., 2008), *Toxorhynchites splendens* (Pattanakitsakul et al., 2007), and *Anopheles minimus* (Rwegoshora, Baisley and Kittayapong, 2000). It is well established that mosquitoes are vectors for numerous pathogens that can cause disease in humans and animals. This has led to significant interest in Ukraine and internationally. Over many years, extensive research has led to the identification of 11 species of arbovirus present in Ukraine, responsible for 25% of seasonal fever diseases (Vynohrad et al., 1994). Among these are West Nile (WN), Batai (Bt), and Sindbis (Sb) viruses, which have a high prevalence and a severe course of disease. The disease may range from a flu-like state to meningitis and encephalitis, with a lethal outcome (Vynohrad I. et al., 1994, 1996). The natural foci of these diseases have been identified in numerous districts, particularly in the steppe zone, where WN, Bt, Sb viruses are prevalent; in the forest zone (woodlands), where Bt and Sb viruses are also found; and in the Transcarpathian Region, where WN and Sb viruses are present. These arboviruses have been identified in numerous species of mosquitoes belonging to the subfamily Culicinae, which are prevalent in Eastern Europe. *Aedes communis*, *Ae. punctor*, *Ae. vexans*, *Ae. cantans*, *Ae. excrucians*, *Ae. caspius*, *Culex pipiens*, and *Culiseta annulata* (Vynohrad et al., 1996) are among the species that are likely to participate in arbovirus circulation in the natural foci. This explains the

maintenance of the tight epidemiological situation in these territories.

The mosquito densovirus is capable of replicating within the mosquito, exhibiting a wide tissue tropism. It affects all phases of ontogenesis (Kuznetsova and Buchatsky, 1988; Buchatsky, 1989). Unlike arboviruses, which also replicate within the mosquito's salivary gland cells, the mosquito densovirus is harmless to humans and vertebrates (Lebedinets, Vasi'ieva and Buchatsky, 1976; Vasil'eva et al., 1990). It is well established that the simultaneous infection of an insect with different viruses is often accompanied by the phenomenon of interference, whereby the reproduction of one or both viruses is suppressed in the insect's body (Kelly, 1980). Consequently, it was reasonable to investigate the results of concurrent infection with an arbovirus and a mosquito densovirus.

The aim of the study was to investigate the nature of the interaction between arboviruses and an entomopathogenic densovirus and to determine the effect of the latter on the ability of the mosquito to transmit arboviruses. The overall aim was to evaluate the possibility of using densovirus as a biological agent for unspecific prophylaxis of arboviral infections.

Materials and methods. The following arboviruses were used in this study: West Nile virus (Flaviviridae, *Flavivirus*, antigenic complex of Japanese encephalitis virus), Batai virus (Peribunyaviridae, *Orthobunyavirus*, antigenic complex of Bunyamwera virus), and Sindbis virus (Togaviridae, *Alfavirus*, antigenic complex of Western equine encephalomyelitis virus), as well as densovirus of blood-sucking mosquitoes (Parvoviridae, *Brevidensovirus*). The mosquito *Aedes aegypti* from a laboratory population was used as a model vector. This species was chosen because of its high sensitivity to various arboviruses and densovirus.

The mosquitoes in the experimental group were infected with densovirus in later larval stages (III–IV) to maximize the number of adult insects that become infected with them. Healthy uninfected females served as controls. A total of 106 uninfected females and 104 female mosquitoes from the larvae previously infected with densovirus were used in the experiments. Females were infected with arboviruses 3–4 days after emergence by blood-feeding using a tampon wetted with 10% viral suspension in hemolyzed mouse blood. On the 10th, 15th, and 20th day after infection, the following parameters were determined:

(1) infective titer of arbovirus in mosquitoes;

(2) identification and localization of arbovirus in the salivary glands;

(3) the ability of the infected mosquitoes to transmit the arbovirus through a blood meal.

The quantity of arbovirus present in the suspension utilized for infection and in the infected mosquitoes was quantified by titration with a biological assay utilizing mice. For this method, one-to-two-day-old mice were inoculated in the brain with the viral suspension or mosquito tissue homogenate. The natural threshold dose for infecting the greatest number of mosquitoes with arboviruses is 0.6 to 3.5 log LD_{50}/0.03 ml. In the experimental conditions employed, the arboviral titers in the suspensions utilized for infecting mosquitoes were respectively 5.9–6.2 log LD_{50}/0.01 ml for WN virus; 5.4–6.0 log LD_{50}/0.01 ml for Sb virus; and 4.5–4.7 log LD_{50}/0.01 ml for Bt virus.

The localization of arboviral antigens in the salivary glands of female mosquitoes was determined using indirect immunofluorescence with glandular preparations. The ability of mosquitoes to transmit arboviruses through a blood meal was evaluated by individually feeding females on white mice. The transmission factor was determined by counting the mice exhibiting specific signs of arboviral infection. The presence of virus in their brains was confirmed by a biological assay.

The results were processed by methods of variation statistics using Statistica v. 9.0. To compare mean values Student's *t*-test was used (Van Emden, 2019).

Results. The amounts of the three arboviruses that had accumulated in densovirus-infected and control female mosquitoes were determined. The infective titers for all three viruses were significantly lower in the densovirus-infected mosquitoes (Table 1).

Table 1 — Influence of mosquito densovirus on arbovirus reproduction in mosquito's organism

Virus	Number of mosquitoes, specimens		Incubation period, days	Infectivity, log LD_{50}/0.01 ml	
	Experiment	Control		Experiment	Control
West Nile	10	10	10	5.0	6.2
	12	15	15	4.0	6.3
	12	15	20	4.1	6.3
Sindbis	10	8	10	4.0	4.8
	10	10	15	4.3	6.0
	10	10	20	4.2	5.8
Batai	12	8	10	3.6	4.2
	15	14	15	3.2	4.4
	15	14	20	3.05	4.6

Note. $P < 0.05$ in all experiments in relation to controls.

It should be noted that the maximal decrease in infectivity for WN and Sb viruses was observed on the 15th day, while for Bt virus, it was on the 20th day. Furthermore, the reduced infectivity of WN and Bt viruses was clearly observed for 10 days of the experiment, while in the controls, it remained almost

unchanged (for WN) or increased (for Bt). In contrast, Sb virus elicited a sharp elevation of the titer on the 15th day, which subsequently stabilized.

The transmission of the virus through blood-feeding occurs only when the virus overcomes the intestinal barrier and reproduces itself in mosquito tissues, including the salivary glands. The success of transmission depends on the accumulation of the virus in the apical cavities of the proximal areas in the glandular lateral lobes in amounts high enough for such transmission (the threshold dose).

To determine the localization of arboviruses in the salivary glands in control *Ae. aegypti* females, immunofluorescence was used. Specific fluorescence initially appeared in the proximal areas of the lateral lobes of the salivary glands in 52–69% of the individuals 10 days after infection with all three arboviruses. On the 15th day, the fluorescence became more intense and spread to all glandular cells in 90% of the mosquitoes in that group. By the 20th day, fluorescence was observed in every preparation.

The specific fluorescence observed in the salivary glands of females in the densovirus-infected group was observed in only some cases 10 days after infection. It spread to individual glandular cells and was less intense than that observed in the control group. By the 15th day, one-third of the females exhibited intense fluorescence in the proximal areas of the glandular lateral lobes, and some mosquitoes exhibited fluorescence throughout the salivary glands. The arboviral antigen was detected in only 8–10% of the females in the experimental group on the 20th day. The experiments with densovirus-infected females indicated a significantly reduced arboviral reproduction in the salivary glands. The accumulation of that virus in large concentrations occurred later and in a lower number of females in those experiments.

The transmission of arboviruses through a blood meal was also significantly lower in the experimental groups than in the control groups throughout the entire observation period (Table 2). The most notable difference was observed on the 10th day for WN virus, with a 6-fold reduction, on the 20th day for Sb virus, with a 10-fold reduction, and on the 10th day for Bt virus, with an 8-fold reduction. It is noteworthy that in the control group, an increase in the transmission factor was observed from the 10th day to the 20th day. In contrast, in the females infected with densovirus, the maximal ability for transmission was observed on the 15th day, followed by a subsequent decrease on the 20th day.

For instance, there has been a more than six-fold reduction in the transmission ability of Sb virus between the 15th and 20th day. A previous infection with densovirus had the least effect on arboviral transmission through a bite on the 15th day.

The infection with Sb virus led to the latest manifestation of the ability to lower transmission. It may be associated with slower reproduction of the virus and its lower accumulation in the female's salivary glands.

Table 2 — Influence of mosquito densovirus on arbovirus vector capability

Virus	Number of mosquitoes, specimens		Incubation period, days	Vector capability, %	
	Experiment	Control		Experiment	Control
West Nile	10	10	10	6.4	38.5
	15	15	15	38.2	85.7
	12	15	20	12.0	86.9
Sindbis	10	10	15	44.5	67.4
	10	10	20	7.5	75.6
Batai	12	8	10	2.0	16.6
	15	15	15	28.6	57.0

Note. $P < 0.05$ in all experiments in relation to controls.

Discussion. The results presented above provide compelling evidence for the interference between the entomopathogenic mosquito densovirus and arboviruses. In laboratory experiments, the suppression of reproduction of WN, Sb, and Bt viruses by densovirus results in a significant decrease in their infective titers and a reduction in the transmission factor during blood-feeding. Therefore, the interference of the densovirus with the studied viruses reduces the vector capacity of bloodsucking mosquitoes as transmitters of arboviral infections. The preparation Viroden, which was developed based on the densovirus (Buchatsky, et al., 1987), has demonstrated high efficacy in regulating the number of mosquitoes in both laboratory and field settings (Lebedinets et al., 1978; Kuznetsova and Buchatsky, 1988; Suchman et al., 2006; Carlson, Suchman and Buchatsky, 2006). The mosquito densovirus and the preparation Viroden based on it are completely safe for vertebrates (Lebedinets, Vasi'ieva and Buchatsky, 1976; Vasil'eva et al., 1990). The use of this drug can significantly reduce the vector potential of carriers of arboviral infections.

In light of the mounting challenges posed by climate change, the findings of this research offer promising avenues for addressing the pressing concerns of protecting people and animals from the real biological threats posed by Dengue, Zika, West Nile fever, Chikungunya viruses, and other transmissible mosquito-borne pathogens. As a consequence of rising temperatures, these diseases are spreading to new territories of the European Union (Jacob et al., 2018) and Ukraine (Ecodia, 2020; Vynograd and Shul, 2021). This represents a genuine threat of epidemics and epizootics of new, particularly dangerous diseases in these areas, which

necessitates the intensification of scientific research for the creation of new approaches to biological threat protection.

Conclusions. The suppression of reproduction of West Nile, Sindbis, and Batai viruses in the mosquito body by the mosquito densovirus results in a significant decrease in their infective titers, as well as a reduction in the transmission factor during blood-feeding in laboratory experiments.

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