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SOME BIOLOGICAL PROPERTIES OF CHLORIRIDOVIRUS FROM *CULISETA* MOSQUITOES (DIPTERA: CULICIDAE)

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Summary. Two strains of mosquito iridescent viruses (*Chloriridovirus*) were isolated in Ukraine from seek larvae of bloodsucking mosquitoes *Culiseta annulata* (Schrank, 1776) and *Culiseta morsitans* (Theobald, 1901). Electron microscopic study of tissues from these larvae revealed icosahedral virus particles ranging from 180 nm to 5 nm in diameter, containing dense, pleomorphic nucleotides. Viruses were assembled in the cytoplasm within spherical virosomes. Both viruses contained DNA and 11 polypeptides with molecular weights varying from 16 kD to 98 kD. A DNA restriction analysis of both strains of mosquito chloriridovirus of the genus *Culiseta* and cloning of fragments of their genomes by genetic engineering methods was performed. Isolated strains were sensitive to ultraviolet insolation and heating and were stable to organic solvents such as ether and chloroform. Both isolated strains grew well in mosquito (*Aedes aegypti*, *Aedes pseudoscutellaris*, *Aedes albopictus*) and in Lepidoptera (*Euxoa scandens*, *Antheraea pernyi*) cell lines. A close antigenic relationship has been found between isolated strains and *Chloriridovirus* from *Aedes cantans.* Some antigenic relationship was also demonstrated between isolated strains and still unclassified iridovirus from carp (*Cyprinus carpio*). These findings imply that both strains share some similarity in structural and biochemical characteristics and belong to the *Chloriridovirus* genus of the Iridoviridae family

Keywords: electron microscopy, carp, Iridoviridae

Introduction. The family Iridoviridae consists of six genera: *Lymphocystivirus*, *Iridovirus*, *Megalocytivirus*, *Decapodiridovirus*, *Ranavirus*, and *Chloriridovirus* (Chinchar et al., 2017). The main members of the genus *Chloriridovirus* are isolated from mosquitoes, midges, and some moths (Wong et al., 2011; Piegu et al. 2013; Huang et al., 2015). The type species for the genus *Chloriridovirus* is IIV-3, which was isolated from *Aedes taeniorhinchus* larvae (Chinchar et al., 2017). Mosquito chloryridoviruses are among the most important pathogens of mosquitoes worldwide (Muttis, Micieli and García, 2023). They were found in *Aedes* mosquito larvae in Czechoslovakia (Weiser, 1965), USA (Clark, Kellen and Lum, 1965), Great Britain (Tinsley et al., 1971), Kazakhstan (Torybaev and Dubitskiy, 1971), Ukraine (Buchatsky and Sheremet. 1974), Argentina (Muttis et al., 2012), and other countries (Williams, 2008).

In Ukraine, up-to-date mosquito chloryridovirus was found among 13 species of *Aedes* mosquito larvae (Buchatsky, 1975; Buchatsky and Sherban, 1976; Buchatsky, Victorov-Nabokov and Sheremet, 1976). In addition to the genus *Aedes*, chloriridovirus was also isolated from blood-sucking mosquitoes of genera *Culex* (Buchatsky, Victorov-Nabokov and Sheremet, 1976; Muttis et al, 2012), *Psorophora* (Chapman et al., 1966), *Anopheles* (Huang et al., 2015), and from non-blood-sucking mosquito *Mochlonyx culiciformis* (Buchatsky, Victorov-Nabokov and Sheremet, 1976). The first record of chloriridovirus isolated from the genus *Culiseta* was in Ukraine (Buchatsky, Victorov-Nabokov and Sheremet, 1976; Buchatsky, 1977).

Therefore, our study aimed to compare the antigenic, ultrastructural, and other characteristics of the two strains of chloriridovirus the genus *Culiseta* and made a comparison between them.

Materials and methods. *Cell and viruses.* Mosquito tissue cultures were grown at 27 °C in medium KC-13, and lepidopteran cells — in medium JC-7, containing 5% and 10% fetal calf serum, respectively.

An isolate of mosquito chloriridovirus, obtained from *Culiseta annulata* (CaChIV) was passaged 27 times in honeycomb moth *Galleria mellonella* larvae as was described Buchatsky, Kaniuka and Lebedinets (1976). Following infection and incubation for 48 h cells and debris were removed by centrifugation and the released virus in the culture medium was stored at a temperature 4 °C.

Both strains were found in different geographical settings of the country. The *Culiseta morsitans* chloriridovirus (CmChIV) was isolated from mosquito larvae in natural small ponds in Sumy Region; CaChIV — in the vicinity of Kyiv.

Infection trials. The susceptibility of honeycomb moth larvae *Galleria mellonella* to mosquito chloriridovirus following intrahaemoceal injection (0.02 ml 10-fold serial dilutions of homogenates from infected larvae) provides a good model for infection trials. The honeycomb moth larvae were examined for the presence of the virus on 14th day of post-infection. The presence of the virus was confirmed by the method of 'colored pellets' (Buchatsky, Kaniuka and Lebedinets, 1976). Infectious titers of chloriridoviruses were counted as described in Reed and Mench (1939).

Electron microscopy. For negative contrast electron microscopy triturated tissues of both infected *Culiseta* species were adsorbed to cooper grids and negatively

stained with 2% phosphotungstic acid (PTA) adjusted to pH 6.8 with 1 M KOH.

For thin layer electron microscopy infected larvae were fixed in phosphate-buffered 2.5% glutaraldehyde, post-fixed in 1% osmium tetroxide, dehydrated in graded alcohols, and embedded in 812 Epon resin.

Ultra-thin sections were double stained in uranyl and lead citrate. Particles exhibiting a hexagonal outline were measured vertex to vertex. Examinations were performed with a JEMB-100 B electron microscope.

Purification of viruses. Both strains of mosquito chlorirodovirus were purified as described previously (Buchatsky, Kuznetsova and Prima, 1983).

Constants of sedimentation. The viruses were resuspended in 0.05 tris-HCl (pH 7.2) in different concentrations (from 0.5 mg/ml to 1 mg/ml) and centrifuged in 'MOM 3175' under 5,000 rpm at a temperature 20 °C.

Constants of sedimentation were measured as described in Bowen (1976).

Histology. Infected larvae were placed into Bouin's fixative and neutral formalin and allowed to fix for 24 h at room temperature. They were then stored at a temperature 4 °C for 1 week before being dehydrated and processed for paraffin embedding.

Tissue sections were stained with hematoxylin and eosin.

Sensitivity to the organic solvents, UV, and heat. Susceptibilities of isolated strains to organic solvents were tested after they were mixed (1:1) and incubated at a temperature 4 °C for 12 h. After this ether or chloroform were moved off and viruses were titrated on *Galleria mellonella* larvae. Susceptibility of chloriridovirus to ultraviolet was tested on viruses with a concentration of 1 mg/ml contained in Petri dishes at a distance of 100 cm from ultraviolet source (OMB-30) power 1800 WA. Infectious trials were performed as above.

Antigenic relationships. The antiserum to chloriridoviruses were prepared as described previously (Filenko, Lebedinetz and Buchatsky, 1995). Antigenic relationships were tested by using the Ouchterlony method (Bailey, 1996).

SDS-PAGE analysis of virion polypeptides. Viruses were boiled for 2 min and then subjected to SDS-PAGE on a 12% polyacrylamide (Buchatsky, Kuznetsova and Sherban, 1982). The gel was run for 5 h at 11 mA. The polypeptide bands were stained with 1% Coomassie Blue. The gel was treated twice with dimethyl sulfoxide for 30 min and then washed with water.

DNA cloning. Mosquito chloroiridovirus DNA was isolated and purified by the phenol-detergent method (Buchatsky, Kuznetsova and Prima, 1983) and by Wesley and Tuthill (1982). For the treatment of DNA preparations with EcoR1 and Msp1 restriction enzymes, an incubation mixture of 20 μl was used, containing 1 μg of chloroiridovirus DNA, a buffer solution, and 5 units of EcoR1 or Msp1 restriction enzymes. The reaction mixture was incubated at a temperature 37 °C for 2 h. To obtain fragments with a known molecular weight (markers), phage λ DNA was treated with EcoR1 and EcoRV restriction enzymes. Electrophoretic separation of DNA fragments was carried out in a 0.7% horizontal agarose gel (130×84×4 mm) at a constant current of 10 mA and a voltage of 30 V for 17–18 h until the band of bromophenol blue reaches the end of the plate. The molecular mass of DNA fragments was determined using the program Scheffer and Sederoff (1981). Cloning of *Culiseta annulata* chloroiridovirus DNA was carried out at the Pst1 site in *E. coli* bacteria. The shuttle plasmid PMK 419, which can replicate in *E. coli* and *Bacillus subtilis* (Sullivan, Yasbin and Young, 1984), was used as a vector.

Results. *Clinical signs.* Infected mosquito larvae displayed classical clinical signs associated with the iridoviral infection. In sunlight, only the thorax of infected larvae was iridescent (from bluish to violet) because of the dark natural color of larvae of these species. The larvae became violet at an advanced stage of the infection and were hardly visible against the black leaf litter at the bottom of the pond. Before death, infected larvae became sluggish and inactive. Their body was neither swollen nor had any cysts, which are common in protozoan infections found in infected tissues. Neither bacteriological nor parasitological examination yielded any significant findings.

Electron microscopy. A preliminary electron microscopical examination of crude hemolymph extracts from infected mosquitoes revealed the presence of icosahedral virions (Fig. 1).

Similar virions were also detected in various tissues from mosquitoes infected with both viral strains (Fig. 2).

When calibrated relatively tomato mosaic virus, the size of hexagonal virions was estimated as 185 ± 5 nm (corner to corner) and 165 ± 5 nm (facet to facet) in the lest compressed direction. The size and cytoplasmic position of the particles together with icosahedral capsid presumptively identified them as members of the family Iridoviridae.

In thin sections of infected larvae, no major differences were noted for each virus strain in different mosquito hosts. Within the infected cells, virions were observed in different stages of assembly (namely capsid fragments, and incomplete and complete capsids).

Histological findings. The most severely affected tissues of mosquitoes were fat bodies and hypoderma. All larvae with overt infection died before pupation. The fat bodies contain reduced supplying substances. A great number of cytoplasmic organelles were destroyed. Infected cells contain also big inclusions with oval or irregular forms.

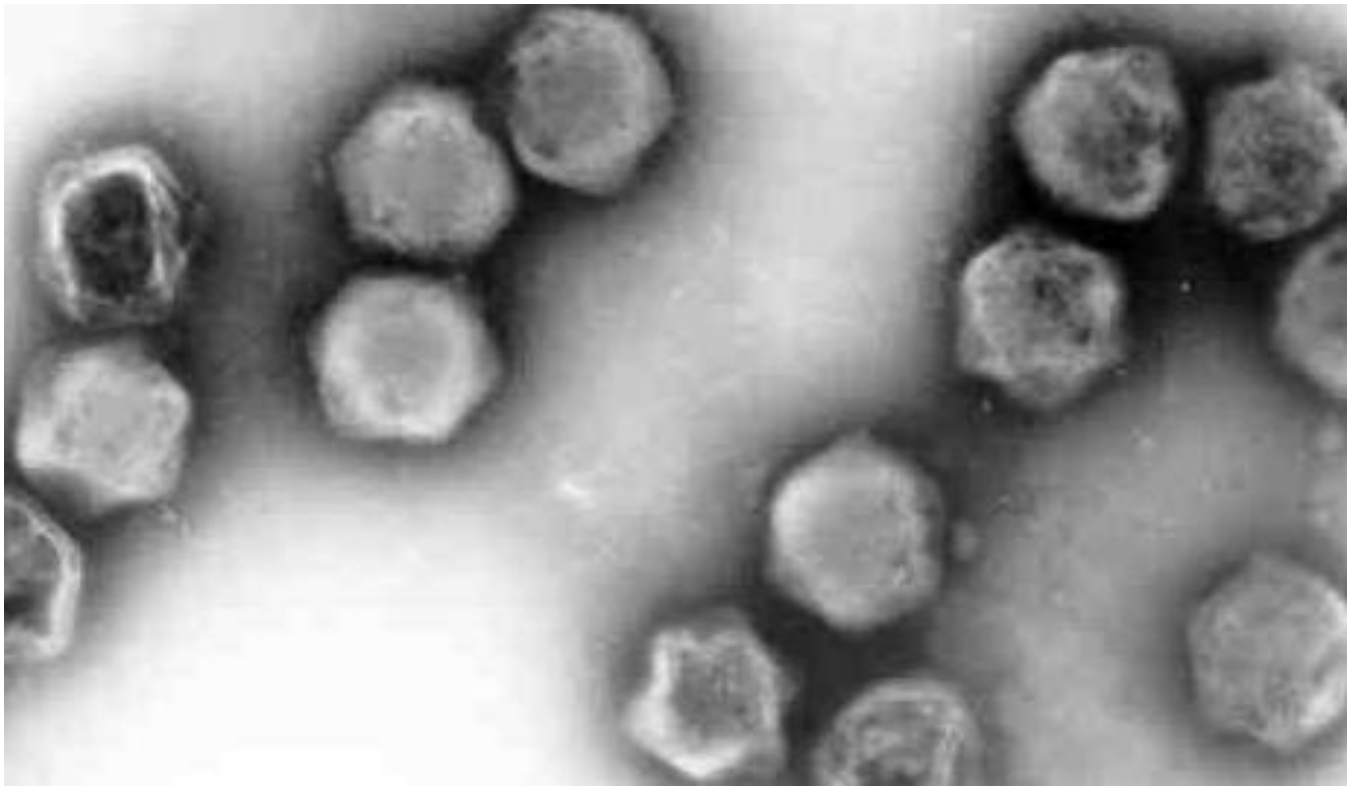

Figure 1. Virus particles of *Culiseta morsitans* chloriridovirus. Negative contrast with uranyl acetate. Virus particles range from 180 nm to 5 nm in diameter.

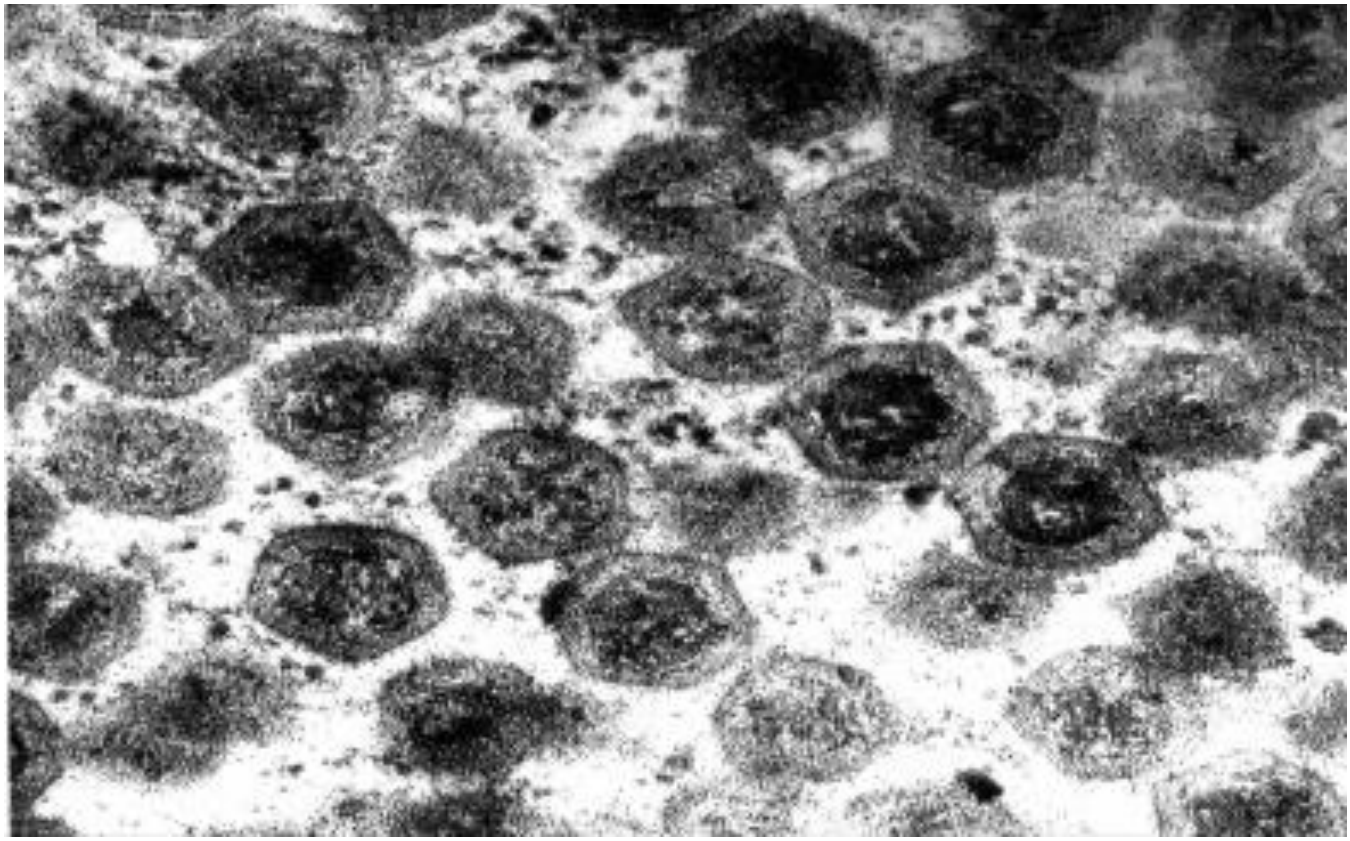

Figure 2. Chloriridovirus particles in the cytoplasm of infected fat body tissue of *Culiseta annulata*. Virus particles range from 180 nm to 5 nm in diameter.

Viral growth in cell culture. Cytopathic effect was observed in cultured *Aedes* mosquito cells 7 days after inoculation with diluted viral suspension. Small dense grayish patches were first seen in the cell's monolayer, which developed into plaques with a border formed by a compact layer of spherical cells, containing refractile cytoplasmic inclusions.

At high inoculum concentrations (> 100 ID_{50}/ml) cytopathic effect was seen in all types of cells in 2–4 h after inoculation. Well-defined syncytia were formed in the infected cells (Fig. 3).

Growth media withdrawn from the cultured cells infected with low concentrations of the virus were titrated on the honeycomb moth larvae.

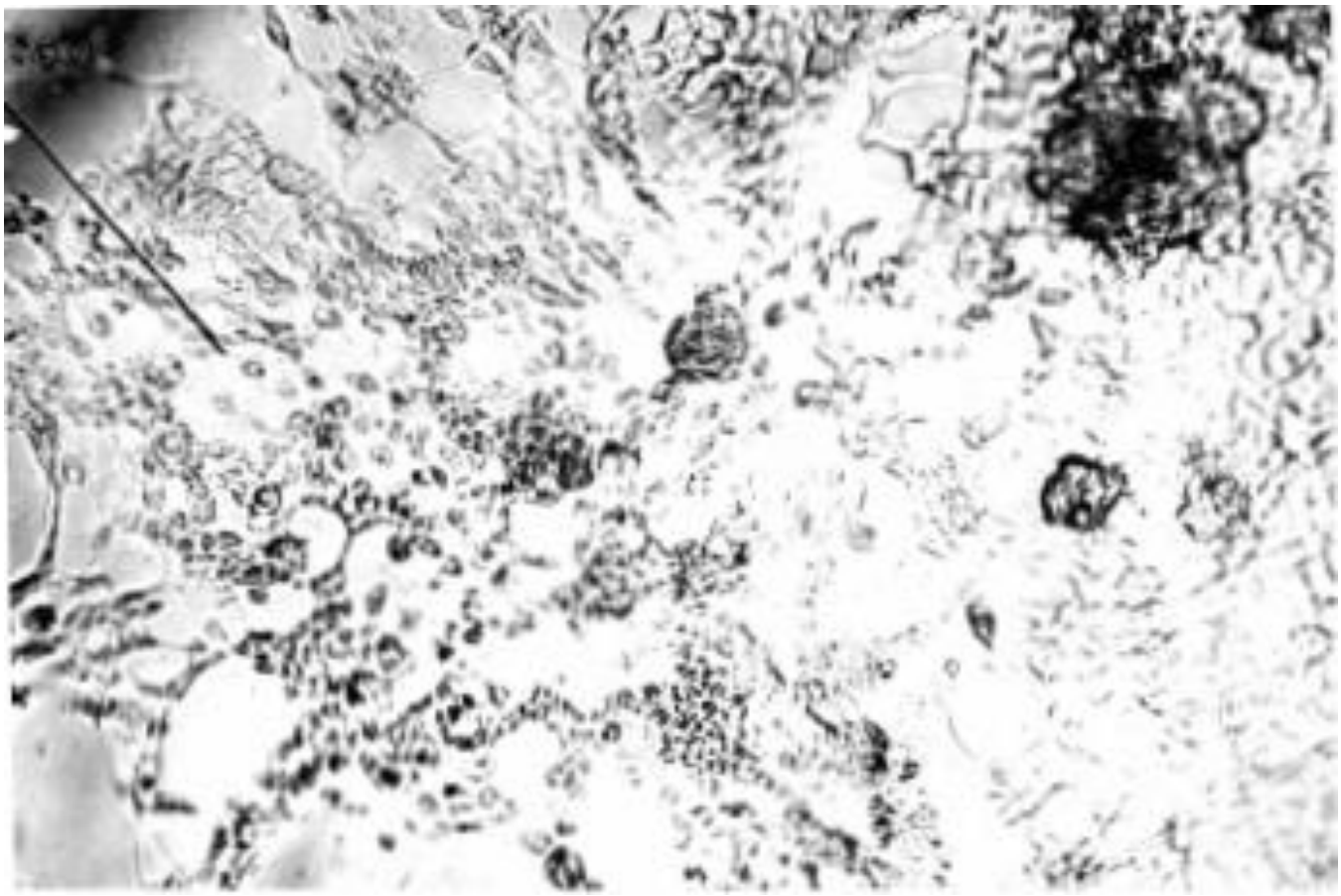

Figure 3. Cytopathic effect of *Culiseta annulata* chloriridovirus (100 ID_{50}/ml) in *Aedes aegypti* cells 4 h after inoculation.

Titration experiments revealed the reproduction of both strains of mosquito chloriridovirus in all cell lines (Table 1). Although the levels of virus reproduction were similar in all cell lines, the cytopathic effect was observed only in *Aedes aegypti* cell line.

Properties of viruses. Both strains of mosquito chloriridovirus purified from infected larvae were dark blue colored. They had the same (2,800 S) constant of sedimentation. Molecular weights were calculated by the following formula:

$$S_{20}, w = 1.114 \times 1O \times M^{0.175},$$

where S — constant of sedimentation at a temperature 20 °C,

M — molecular weight.

Nucleic acids isolated from both strains reacted positively with diphenylamine and negatively with orcin suggesting that viruses contain DNA. Isolated DNA of both strains had a constant of sedimentation 70 S, their points of melting were 86.3 °C and their buoyant density in CzCl solution was 1.7043 g/cm^3.

Table 1 — Titration of the cultural media from insect cells infected with chloriridoviruses on *Galleria mellonella* larvae (lg ID_{50}/ml)

No.	Cell lines	CmChIV	CaChIV	CPD	P
1	*Aedes aegypti*	4.7	5.0	+	> 0.05
2	*Aedes pseudoscutellaris*	5.0	4.7	–	> 0.05
3	*Aedes albopictus*	4.0	4.3	–	> 0.05
4	*Euxoa scandens*	4.3	4.2	–	> 0.05
5	*Antheraea pernyi*	4.3	4.1	–	> 0.05

Table 2 — Molecular weights (kDa) of polypeptides isolated from chloriridovirus of *Culiseta annulata*, *Culiseta morsitans* and *Aedes cantans*

CaChIV	CmChIV	AcChIV
98	98	95
89	89	87
74	74	77
50	50	62
40	40	54
36	36	36
28	28	28
25	25	22
20	20	16.5
17	17	
16	16	

Antigenic relationships. In gel immunodiffusion tests only one line of precipitation was detected in homologous as well as heterologous systems (Fig. 4). These results suggest a close antigenic relationship between *Culiseta* and *Aedes* chloridoviruses as well as between both strains of *Culiseta* viruses and chloridovirus from *Cyprinus carpio*.

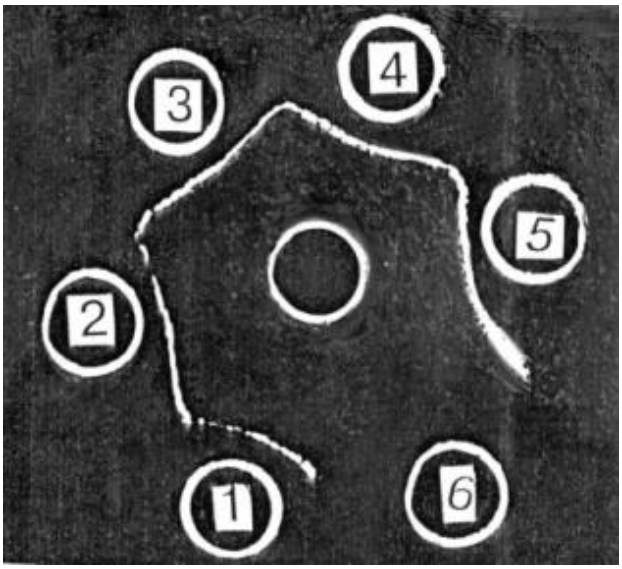

Figure 4. Antigenic relationships of two strains of *Culiseta* chloriridovirus with chloriridovirus from *Aedes* genera. Cloriridoviruses from: 1 — *Culiseta annulata*, 2 — *Culiseta morsitans*, 3 — *Aedes cantans*, 4 — *Aedes flavescens*, 5 — iridovirus from *Cyprinus carpio*, 6 — tissue homogenate from *Galleria mellonella* larvae. Central hole — antiserum to *Aedes cantans* chloriridovirus.

The results of passive agglutination also indicated that carp (*Cyprinus carpio*) iridovirus gave a positive reaction with antisera to chloriridovirus from *Aedes cantans* in titers $^1/_{16}$–$^1/_{64}$, but in homologous systems were higher titers — $^1/_{512}$–$^1/_{1,024}$.

Resistance of mosquito chloriridovirus to UV-light, heat, and organic solvents. Purified chloriridovirus from both strains lost their infectivity after 15 min exposure under UV light (Fig. 5). Non-purified chloriridovirus (supernatant of crude homogenates from infected larvae) appeared to be more resistant and lost the infectivity only after 30 min exposition.

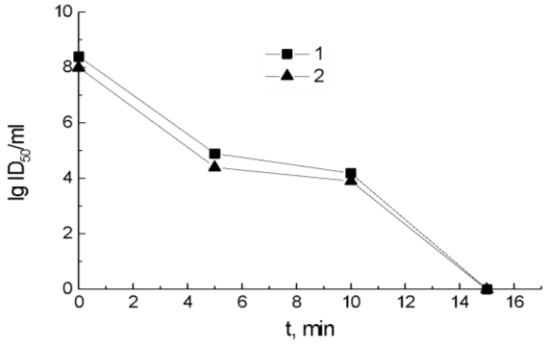

Figure 5. Influence of UV irradiation on two strains of *Culiseta* chloriridoviruses: 1 — CaChIV, 2 — CmChIV.

Both strains of *Culiseta* chloridovirus were not sensitive to ether and chloroform. Titers of their infectivity remained stable after the treatments with these organic solvents (Table 3).

Table 3 — Effect of ether and chloroform on the infectivity of chloriridoviruses from *Culiseta annulata* and *Culiseta morsitans*

Organic solvents	Infectious titers, lg ID_{50}/ml		P
	CaChIV	CmChIV	
Ether	8.9	8.8	> 0.05
Chloroform	8.8	8.9	> 0.05
Control	8.9	9.0	

One of the important characteristics of viruses is the level of their resistance to heating. We determined that both strains of *Culiseta* chloriridovirus were sensitive to high temperatures (60 °C). Incubation of viral suspension at a temperature 60 °C for 30 min resulted in a significant decrease of the infectivity, prolonged incubation (60 min) led to complete inactivation of viruses (Fig. 6).

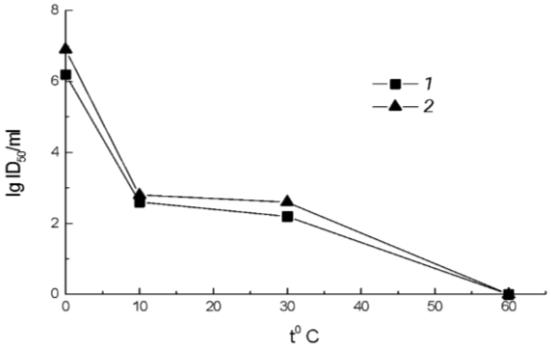

Figure 6. Infectivity of *Culiseta* chloriridoviruses at a temperature 60 °C: 1 — CaChIV, 2 — CmChIV.

The study of electropherograms of DNA fragments of chloroiridovirus from mosquitoes *Culiseta annulata* and *Culiseta morsitans* showed the identity of hydrolysis and mobility of DNA restrictions for these strains of iridoviruses. Upon hydrolysis of DNA from both strains of chloroiridovirus with EcoR1 restriction enzyme, 26 DNA fragments ranging in size from 24.7 kb to 1.7 kb were detected. After DNA treatment with Msp1 restriction enzyme, 31 fragments with sizes from 13.5 kb to 1.7 kb were detected. The sizes of the DNA of the two strains of chloroiridovirus obtained as a result of restriction analysis range from 148 kb to 154 kb, corresponding to approximately $110–115\times10^6$ Da. Thus, the results of DNA restriction analysis of chloriridovirus of *Culiseta annulata* and *Culiseta morsitans* mosquitoes allow us to conclude about a significant degree of homology of these strains of chloriridoviruses of blood-sucking mosquitoes. To clone the genome of the chloroiridovirus of *Culiseta annulata* mosquitoes, DNA was treated with Pst1 restriction enzyme. Plasmid RMK 419, which was used as a vector, had one restriction site for Pst1. Pst1 ligation of the chloroiridoviral DNA restriction and plasmid resulted in the formation of a recombinant DNA molecule that was used to transform competent *E. coli* cells. When the transformants were transferred to the medium containing IPTG and XGal, the colonies with the chloriridoviral DNA insert were colored white, and the colonies with the original plasmid were colored blue.

Treatment of the original plasmid with Pst1+Hind 111 restriction enzymes leads to the formation of two DNA bands during electrophoresis, and for the recombinant plasmid — to cleavage of the *Culiseta annulata* chloroiridovirus DNA fragment inserted at the Pst1 site and two fragments characteristic of the original plasmid. As a result of the work carried out, an insert of iridovirus DNA with 1.5 kb was isolated, which can be used in the construction of diagnostic probes.

Discussion. We report here about the biological properties of two strains of viruses isolated from the mosquito larvae of the genus *Culiseta*. The size and morphology of these viruses, the presence of lipid envelope, CPE in cultured cells from different insects, and the presence of DNA, are consistent with *Chloriridovirus* genus (Devauchelle et al., 1985). As shown (Stoltz, 1971, Wagner et al., 1973), chloriridoviruses have bigger sizes and contain fewer polypeptides in comparison with other iridoviruses of insects. Iridovirus from *Tipula paludosa* contains 28 polypeptides with molecular weight varying from 17.5 kDa to 30 kDa (Krell and Lee, 1974). Iridovirus from *Sericestis pruinosa* contains 22 polypeptides (Elliot, Lescott and Kelly, 1977), iridoviruses from *Simulium* and *Chilo* — 25 polypeptides (Devauchelle et al., 1985). In contrast, the mosquito chloriridoniruses isolated from *Aedes taeniorhinchus* or *Aedes cantans* contain only 11 (Wagner et al., 1973) and 9 polypeptides (Buchatsky, Kuznetsova and Sherban, 1982), respectively.

Our results described above show that *Culiseta* chloriridoviruses contain 11 polypeptides with molecular weights varying from 16 kDa to 98 kDa. Some of these polypeptides may possess toxic activity for cells as was shown for other iridoviruses (Cerruti and Devauchelle, 1979). We observed a toxic effect in all types of cell lines infected with large doses of *Culiseta* chloriridoviruses (> 100 ID_{50}/ml). No effect was detected for small doses of both strains of chloriridoviruses, except for *Aedes aegypti* culture.

Results of our experiments on the resistance of two strains of *Culiseta* chloriridovirus to heating are well consistent with data obtained for other iridoviruses. An increase in incubation temperature led to the loss of iridovirus infectivity (Carter, 1975).

Iridoviruses are sensitive to exposure to light (Klump, Beaumais and Devauchelle, 1983). However, some protectors may increase viral resistance to the damaging effect of UV light. Higher resistance to the UV light observed for *Culiseta* chloriridovirus maintained in crude homogenates from infected larvae in comparison with purified viral preparations suggests that host tissues (cells) may contain protective factors.

Our experiments show that both strains of *Culiseta* chloriridovirus are resistant to ether and chloroform. As hypothesized (Balange-Orange and Devauchelle, 1982) such resistance of iridoviruses to organic solvents could be a result of high content of phosphatidylinositol (acid phospholipids) found in viral capsid. Alternatively (Kelly and Vance, 1973; Kelly et al., 1979) it is speculated that the lipid bilayer is located within the virion's capsid and, therefore, is not accessible to solvents.

In our experiments, close antigenic relationships between both strains of *Culiseta* chloriridovirus, *Aedes cantans* chloriridovirus and iridovirus from carp were shown. Antigenic relationships among different genera of iridoviruses are poorly investigated. It was shown (Cunningham and Tinsley, 1968) that mosquito chloriridovirus is not antigenically related to small iridoviruses of insects from the genus *Iridovirus*. No antigenic difference was found between the two strains (R and T) of chloriridovirus from *Aedes taeniorhynchus* (Hall and Love, 1972). Antigenic relationships among small iridoviruses vary from none to close. Mosquitoes are also susceptible to invertebrate iridescent virus 6 (IIV-6) which was originally isolated from *Chilo suppressalis* (Lepidoptera: Pyralidae) (Marina et al., 1999, 2003) which is the type species for the genus *Iridovirus* (Chinchar et al., 2017).

At present no similarity between mosquito chloriridoviruses and fish iridovirus has been reported. Our previous results (Yaremenko and Buchatsky, 2003)

demonstrate that still unclassified iridovirus from carp shares many similar biological properties with chloriridoviruses and like chloriridovirus, grows well in the honeycomb moth larvae. In addition, using PCR it was shown that the chloriridovirus of mosquitoes can multiply in the body of carp (Rud and Buchatskyi, 2009). We suggest that one of the possible candidates for the role of the etiological agent of gill necrosis of carp may be mosquito chloriridovirus. We base our suggestion on the similarity in biological, structural, and antigenic properties found between two strains of mosquito *Culiseta* chloriridovirus and carp iridovirus.

Conclusions. A close antigenic relationship has been found among two isolated virus strains from *Culiseta* and *Chloriridovirus* from *Aedes cantans.* Some antigenic relationship was also demonstrated between isolated strains and still unclassified iridovirus from carp (*Cyprinus carpio*). These findings imply that both chloriridovirus strains from *Culiseta* mosquitoes share some similarity in structural and biochemical characteristics and may belong to the genus *Chloriridovirus* from the family Iridoviridae.

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