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VIRAL INFECTIONS OF REPTILES: A REAL THREATFOR HEALTH OF HUMANS AND WARM-BLOODED ANIMALS

Статья проясняет необходимость обратить внимание на вопрос распротранения вирусов среди рептилий, которые могут представлять серьезную угрозу для здоровья людей и животных. Проведены пилотные исследования по получению чувствительной системы для детекции вирусов рептилий, которые в дальнейшем будут использованы для рарзработки диагностических методов.

This paper deals with problems concerning the spreading of reptilian viruses which may be a serious threat for human and animal health. Preliminary studies have been carried out aiming the elaboration of a sensible test system permitting to detect reptilian viruses and to develop diagnostics methods.

Introduction. Viral diseases of poikilotherm animals as well as viral agents transmitted or/and persisted in poikilotherm reservoir animals are now an almost unstudied and actual problem of contemporary virology. Investigations of reptilian pathology being today carried out belong to relatively new scientific trends which have become to develop rapidly during these last years [2, 13]. Research of disease agents able to infect both cold- and warm-blooded hosts is mostly focused on reptiles participation in epidemiology of diseases caused by viruses belonging to families mentioned above. It should also emphasize the importance of reptile viral disease research for veterinary specialists dealing with animal patients kept at home, the studies in this field being also essential from the scientific point of view concerning taxonomic investigations and virus evolution. In Ukraine there are almost no investigations of viral diseases whose victims and/or vectors are reptiles.

Object of this work is to draw attention to the importance of reptile viral infection problem as a possible source of virus spreading among animals and humans. In addition, the aim of our experimental research is to elaborate some protocols permitting to obtain virus susceptible cell systems adequate for isolation of reptile viruses.

Today representatives of all *Reptilia* classes are known to be possible intermediate hosts or reservoirs of different pathogens [2, 4, 5, 13]. Numerous viruses isolated from these animals have been described including those belonging to the arbovirus group (togaviruses, flaviviruses, rhabdoviruses, and bunyaviruses) transmitted by arthropods and causing infections of humans as well as of other mammals and birds [4,6,10]. Numerous studies prove arboviruses to possess enormous pathogenic possibilities. During last two-three decades, a lot of jointed demographic, socio-economical, and ecological factors led to a unique combination of previously absent conditions being especially favorable for increasing of epidemic potencies for numerous arboviruses (yellow fever, West Nile, dengue fever, and Chikungunya viruses) as well as for their spreading on territories where they had been previously absent, such spreading being accompanied by devastating epidemics [3,4]. The scientific data have already proved different members of the arbovirus group to be able to infect any reptilian species [26]. Under certain temperature conditions, viremia can appear in these animals. The recent studies demonstrate some arboviruses are able to persist in reptilian organisms during the winter period. Such a factor plays an outstanding role in infective epidemiology of diseases caused by these viruses. The West Nile virus (WNV) causes an arboviral disease endemic for Africa, Asia Europe, Europe, and Oceania, the infection being transmitted by mosquitoes of the *Culicidae* family. This virus infects susceptible mammalian species including humans as well as avian species causing the development of meningoencephalitis. The first epizootic outbreak due to this virus activity among birds was registered in the USA (New-York) in 1999 [12]. Before 2002 the virus spread on territories of all states; 120 humans fell ill in the same years, 11 of them died [2]. In 2001 first cases of alligator disease were reported from the state of Florida alligator farms; next year such reports were from farms of Georgia as well as from a Nile crocodile farm in Israel and from Mexico where the disease had been detected among wild crocodiles [2,10]. Until 2005, the disease was registered in states Texas, Louisiana, and Idaho. In Louisiana, 5000 young alligators perished, four cases of human infection among the farm workers have been found [30]. Seropositive individuals were detected in epizootic nidi among wild alligators [2]. The West Nile virus is a typical emergent infection for *Crocodylidae;* it is probably due to introduction of a viral strain being pathogenic for humans, birds, and horses. There are some data proving a high viremia degree in diseased alligators associated with marked virus secretion. Taking into consideration the peculiarities of epizootic outbreaks it is foreseen the infective agent to be spread among sensible *Crocodylidae* species by other ways, not only by transmission. It has been recently reported about parenteral and oral virus inoculation to alligators [13]. These data suggest a possible danger of infection for humans being in direct contact with animal feces and tissues [2,14].

The Japanese encephalitis virus (JEV) has been isolated from Chinese rat snakes (Elaphe rufodorsata) in Korea [15]. And the antibodies to JEV has been found in cobras (Naja naja) in Hong Kong. Transmission studies with Japanese encephalitis virus have shown that lizards can be infected with this virus both by parenteral inoculation and, in some species, by feeding on infected mosquitoes. Infected animals develop viremia, and the development of viremia is temperature dependent [205]. No clinical symptoms were reported in the lizards in that study. Direct virus detection in naturally infected reptiles by isolation in cell culture or by RT-PCR has been described less frequently[13].

Most alphaviruses can infect a wide range of vertebrates, mostly birds and mammals,but several have also been reported in reptiles. Studies on alphaviruses in reptiles have mostly focused on the possible role of these animals for the transmission of alphaviruses to humans and livestock. This has led to a focus on persistence of alphaviruses in reptiles, particularly viral persistence over winter in temperate regions in the absence of mosquito activity. Evidence of infections with eastern equine encephalitis (EEE) virus and western equine encephalitis (WEE) virus, either by isolation or serology, have been reported in a variety of chelonians, lizards (including members of the families Lacertidae, Teiidae, Iguanidae, Agamidae, and Gekkonidae) and snakes (including members of the families Colubridae, Elapidae, and Crotalidae)[10]. Transmission studies have been performed in garter snakes to determine whether the virus can overwinter in snakes, can result in antibody production, and be infectious to mosquitoes. Environmental (body) temperature affects viremia, with no viremia detected in experimentally infected snakes during torpor, and a lag time of several days required to detect the virus after an animal emerges and is warmed. In some experimental studies, a persistent viremia was seen.[10] A transmission study involving subcutaneous injection of spotted turtles (Clemmys guttata) with EEE virus led to the development of viremia and neutralizing antibodies in infected turtles. Subcutaneous infection of Texas tortoises (Gopherus berlandieri) with WEE virus led to high titer viremia over an extended period of time. Viremia was longer at lower temperatures than at high environmental temperatures. None of the transmission studies described any signs of clinical disease in infected reptiles [13].

The investigations carried out by scientists of several universities in Alabama prove the poikilotherm vertebrates may be reservoir hosts of the Eastern equine encephalomyelitis virus (EEEV) [11]. During 2001-2003, females of blood-suckling *Culicidae* mosquitoes belonging to *Culex, Aedes, Culiseta,* and *Uranotaenia* genera were tested as possible EEEV-transmitting vectors. The data obtained show that certain mosquito species of genera *Uranotaenia* and *Culex (U. sapphirina, C. erraticus,* and *C.peccator)* collected during the transmission period (from April to October) prefer poikilotherm vertebrates as their hosts. The species *C.peccator* belongs to the subgenus *Melanoconion,* its members being vectors of alphaviruses [6,11]. During the study period the EEEV was detected in all mosquito species. Poikilotherm animal species were supposed to be possible EEEV reservoirs in South-Eastern territories of the USA, mosquito species *C. peccator* and *U.sapphirina* being found to feed on reptiles and amphibians. There are also publications concerning the EEEV detection in other mosquito species – *Aedes vexans* and *Culiseta melanura* feeding also on reptiles and amphibians; they may be an important link in the chain of the EEEV spreading. According to the data obtained by P.Ya.Kilochyts'ki[8], N.P.Kilochyts'ka, and V.P.Sheremet, the urbanization process has given a stimulus for a new direction of mosquito evolution accompanied by intensive adaptation for existence in new environment conditions. Seven mosquito species including *Aedes vexans* and other members of *Aedes* and *Culex* genera are now considered as potentially dangerous vectors of human and animal infective agents [3]. The mosquito genera being the most important for arbovirus transmission are *Aedes, Culex,* and *Haemagogus* [3,6].

According to the data of official Interpol and CITES statistics, the world volume of illegal market turnover concerning endangered animal species reaches from $8-10 up to $20 milliard annually; it is the second position comparing to drug market gains (forestalling the arm trade ones). [1,16] (table 1).

***Table* 1. Volume of the alive reptiles market turnover (for some countries)**

Country	Period of time, years	Quantity of alive reptiles imported (annually)	Quantity of alive reptiles exported
USA	1989-1997	2,000,000	9,000,000 *
	2002	2,250,000	
EC countries	1992-2002	11,000**	
South Korea	1993	1,400,000***	
Italy	1993	1,300,000	
Ukraine	1999-2009	1,000,000	

*In most cases they are read-eared turtles *(Trachemys scripta elegans);* according to the USA legislation, any species of fresh water turtles cannot be imported, the trade within the country being forbidden if their test diameter exceeds 10 cm; it is a preventive measure against salmonellosis outbreaks, these animals being their common source.

**These data concern only crocodiles and monitors *(Varanus* sp.).

***These data concern only read-eared turtles.

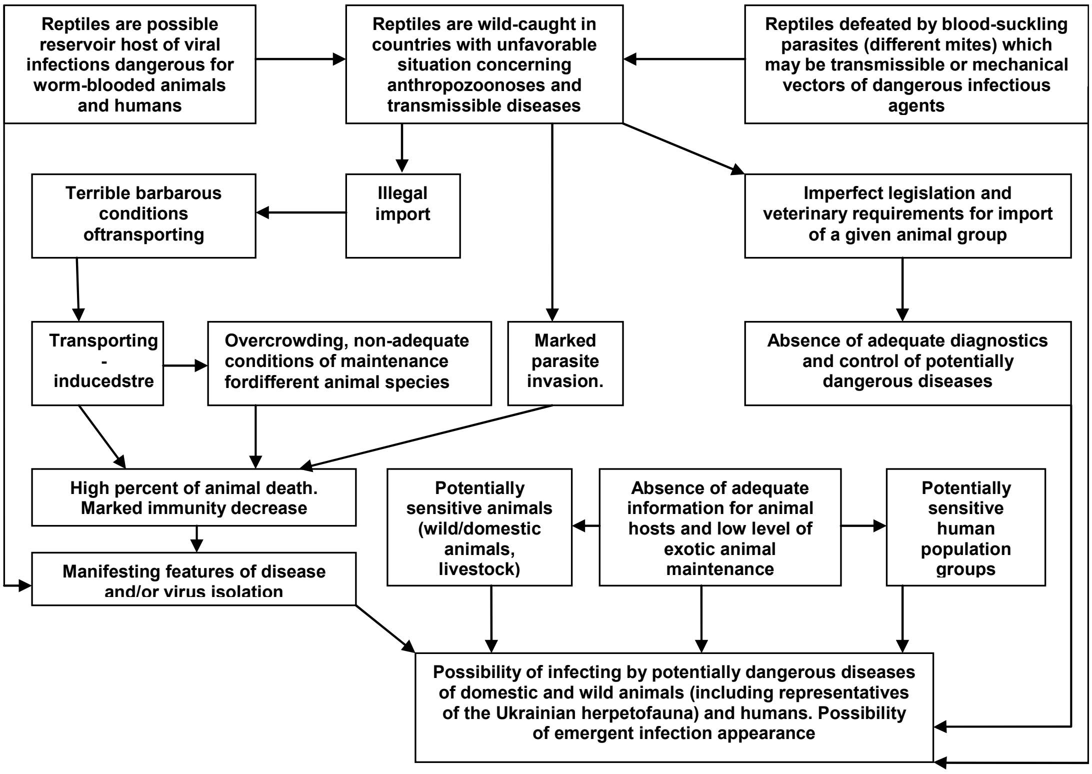
Independent experts consider the annual animal turnover smuggling in Ukraine reaches $200 million [1]. Our country is not only an outlet for such animals, but also their transit, export, and reexport nidus. According to the WWF data, Ukraine is the main transit country concerning wild animal supply from the Western Europe to post-Soviet states and vice versa [1]. The Kyiv Eco-Cultural Center[1] considers more than 10 million of reptiles together with other animals have been illegally imported to Ukraine during last years [1]. According to official statistics, the quantity of arrested illegal imported exotic animal batches in post-Soviet countries become almost twice higher comparing to batches arrested several years ago [16]. The main suppliers of reptiles, amphibians and other exotic animals for Russian and Ukrainian "black market" are countries of South-Eastern Asia, South America, and Africa, most of them being unfavorable from the point of view of dangerous viral infections [16]. Among such infections there are also ones whose vectors and/or intermediate hosts may be also cold-blooded animals (table 2).

***Table* 2. Anthropozoonoses which may be common for cold-blooded, warm-blooded animals, and humans**

Disease and its agent	Hosts and vectors	Disease manifestations in domestic animals	Source of isolates (of reptile viruses)	Disease manifestations in humans
Western equine encephalomyelitis (WEE), a virus of *Togaviridae* family	Birds, squirrels, snakes, horses. Vectors: mosquitoes	Without manifestations in all animals except horses, their lethality being about 30%	Blood samples of tortoises and crocodiles	Encephalomyelitis
Eastern equine encephalomyelitis (EEE), a virus of *Togaviridae* family	Birds, some lizards, and crocodiles Vectors: mosquitoes	Without manifestations in all animals except horses, their lethality being about 95%	Antibodies are detected in tegu lizards; some wild alligatos of Mississippi are also proved to be seropositive	
West Nile fever (VNF), a virus of *Flaviviridae*family	Toads, snakes, crocodiles, monitors, tortoises. Vectors: mosquitoes	Neurologic symptoms Crocodiles: neurologic symptoms, high lethality	Virus is highly pathogenic for crocodiles; high lethality is seen for young animals. The agent was found in 1these animals of all continents.	Fever, neurologic symptoms
Japanese encephalomyelitis, JEM, a virus of *Flaviviridae* family	Lizards, snakes, birds, horses, pigs. Vectors: mosquitoes	Severe encephalitis No symptoms in reptiles	Field isolates from snakes; development of viremia following an infected insect has been eaten	Encephalitis

Besides, new emergent infections appeared during last years are a serious threat not only for human-organized, but also for natural reptile and amphibian populations, so we must pay more attention to researches concerning the viruses mentioned above as probable etiologic agents causing these animals infective diseases [3]. Captive animals caught in wilderness find themselves in contact with new pathogens. Stresses due to capture as well as to transporting and non-adequate life conditions cause significant immune suppression, the animals becoming more susceptible to infections. In addition, animals from different continents are kept very oft together, such conditions being exceptionally favorable for infective agents spreading and their inculcation into new host organisms. Without any doubt, the pathogen evolution takes place, these agents becoming able to infect new hosts in which they have not been earlier detected [2,4,10]. Taking into consideration a low level of animal maintenance by their hosts (the worst situation is seen with exotic species) there is a real threat of new pathogen penetration into the environment. Combination of these factors may lead to pathogen spreading among sensible animals and humans as well as to catastrophic results for local herpetofauna if infected individuals penetrate to local natural animal populations (Scheme 1).

Scheme 1. Possibleways of inter-species transmission of dangerous pathogens and factors favorable for such processes



The infections being the most dangerous for poikilotherm animals are amphibian chytridiomycosis [34] and ranavirosis (it is now included into the OIE list of dangerous diseases [17]) as well as the spreading of the West Nile virus in Mississippi alligator populations in the USA [18]. There are also reports about diseases of other crocodiles species from different continents [3,18,25,30]; insect diseases caused by agents belonging to the Iridoviridae family are found also to infect reptiles [21,26,27]; there are reports on land and water tortoises infecting by the amphibian ranovirus [20,29]. The infections being the most widespread and causative mass mortality of reptiles in collection include: herpesvirus caused infection of tortoises, paramyxovirosis affecting snakes, and inclusion body disease of boas and pythons. About 12 viral agents reproducing in humans and other mammalian species have been successfully inoculated to reptiles during experimental studies [3, 10].

According to Ukrainian veterinary norms, only bacteriological control (to detect the presence of Salmonella) and quarantine during 30 days are required. However, such preventive measures are not sufficient for detection of many dangerous infections widely spread among wild cold-blooded animals. That is why there is an urgent necessity of elaboration of veterinary approaches intended for early diagnostics and including quarantine measures, screening inspection of recently imported or caught animals as well as organization of effective treatment for a given animal group. The problems discussed above become more and more burning not only for persons being reptile hosts and maintaining them as zooculture objects, but especially for those who take part in the realization of programs intended to conserve and re-introduce exotic and rare species as well as in trade regulation, reptile transporting, and elaboration of veterinary norms for this animal group.

We have begun investigations aiming to obtain primary cold-blooded animal cell cultures in prder to elaborate virus-sensitive cell systems necessary for reptile virus isolation. As cell donors African chameleon *(Chamaeleo calyptratus)* and sand lizard *{Lacerta agilis)* were used. Cell suspensions were prepared by cold trypsinization. Now we aim to determine the most favorable temperatures for cell cultivation (29 °C, 37°C) and optimal culture media compositions (based on DMEM and RPMI 1620 media). Chameleon cell cultures (prepared from kidney tissues) were kept 4 days at 28°C before the beginning of cell proliferation. The medium was then substituted after each 3 days of cultivation, different embryonic calf serum concentrations being added (from 10 % up to 20 %). In 10 days a non-confluent cell monolayer was seen, the cells being similar to lengthened spindle-shaped ones (we have not yet found cells of such morphology in the available scientific literature) [8]. The cells which had developed no complete monolayer survived during 29 days, their death causes being unknown. A primary cell culture prepared using *Lazerta agilis* kidneys and testicles survived at 29°C during 16 days.

Conclusions. The analysis of available published data proves the spreading of dangerous pathogens among reptiles, these pathogens being able to cause viral diseases of warm-blooded animals and humans. It is extremely necessary to pay attention to reptilian viruses investigation among Ukrainian reptilian populations to maintain and increase the biosafety in our country.

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FIRST DETECTION OF HOSTA VIRUS X IN UKRAINE

В статье представлены результаты мониторинга X вируса хосты (HVX) в Национальном ботаническом саду Украины им. Н.Н. Гришка. На растениях хосты наблюдали симптомы мозаики и деформации листьев. Присутствие HVX-инфекции в растениях хосты сорта 'Sum of and substance' было подтверждено результатами ELISA, TEM и RT-PCR. HVX был изолирован из больных растений хосты, концентрация очищенного препарата HVX составляла приблизительно 3 мг/мл.

The results of Hosta virus X (HVX) monitoring in M.M. Hryshko National botanical garden of Ukraine (NAS of Ukraine) are presented. Leaf mosaic and deformation symptoms were observed on hosta plants. The presence of HVX in hosta cultivar 'Sum of and substance' was confirmed by ELISA, TEM and RT-PCR. HVX was isolated from diseased hosta. Purification of HVX from hosta leaves yielded about 3 mg/ml.

Hostas or (funkias) are very popular in Ukraine and worldwide, hardy herbaceous perennials grown primarily for their beautiful foliage. Hosta plants generally reache full maturity in 4-8 years, and their size depends on the cultivar [1]. Hosta foliage can be blue, gold (yellow), or green and are often variegated. They are easy to grow and shade-tolerant plants. The plants are low maintenance and are widely available in nurseries and garden centers. In Ukraine hosta settled in private collections, but the biggest collection has been mounted in the National botanical garden by M.M. Hryshko. Certain hosta cultivars are susceptible to a number of viruses, including Hosta Virus X (HVX). Despite its relatively recent discovery, HVX had a significant impact on the hosta industry resulting from the infection and subsequent destruction of many HVX-infected hostas [2]. Symptoms of this virus include mosaic, chlorosis, and necrosis on leaves; severely affected leaves may wither and die. HVX can be transmitted during vegetative propagation of plants and mechanically. Diagnostic laboratories and nursery surveys of symptomatic plantshave confirmed a number of HVX-infected hosta cultivars worldwide [3]. New HVX-infected hosta cultivars continue tobe detected. Investigations of distribution of HVX in Ukraine have not been conducted. The aim of this work was monitoring of HVX in M.M. Hryshko National botanical garden of Ukraine.

Materials and methods. During autumn 2011, the samples of hostas exhibiting virus-like symptoms were collected from M. M. Gryshko National Botanical Garden (NAS of Ukraine). Several samples were collected from symptomless plants. A total 21 samples from 4 hosta cultivars ('Sum and substance', 'Striptease', 'Lady Guinevere', 'Old Faithful') were analyzed for the presence of the virus.

Taking into account serological relation between HVX and PVX (potato virus X) indirect ELISA tests of hosta samples were carried out using antiserum to PVX.

For EM examination, partially purified plant sap was mounted on formvar grids and negatively stained with 2% uranyl acetate. The preparations were viewed under the electron microscope at an instrumental magnification of 20,000.

The method used or virus purification was adapted from those of Goodman (1975) for PVX. The virus was purified by clarification in chloroform, precipitations using polyethylene glycol 6000, and differential centrifugation.

Total RNA was extracted from plant material and subjected to RT-PCR using primers a6448 and s5722 corresponding to nucleotide sequences 6448-6428 and 5722-5742, respectively of HVX from Korea (HVX-Kr) (GenBank Accession No. AJ620114)[1,3].

Results and discussion. The main objective of the present investigation was diagnostic of hosta plantings for infection with HVX in Ukraine. For this purpose four varieties of hosta were tested. Visual monitoring of hosta plantings revealed the presence of plants with symptoms of viral disease. On hosta plants of cultivar 'Sum of and substance' we observed dark, green streaking and puckering along the leaf veins (Fig. 1.). The plants of cultivars 'Striptease' and 'Lady Guinevere' with symptoms of chlorosis and leaf desiccation were collected for farther analysis (Fig. 2,3). Hosta 'Old Faithful' displayed puckering and distortion on leaves (Fig. 4.). According to the literature data the symptoms of HXV-infection can vary depending on cultivar, plant age and growth condition. The viral symptoms also can be confused with symptoms cased by other ethiological agents. To reveal viral etiology of diseases more specific serological methods should be used.