



Indication and Identification of Dengue and Chikungunya Viruses in *Aedes spp.* Mosquitoes Captured in Central America

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The purpose of study was to isolate arboviruses from mosquitoes of different species in the cell culture and to identify them by using molecular and immunochemical techniques.

Materials and methods. Viruses were isolated in C6/36 cell cultures. The pathogens were identified by using enzyme-linked immunosorbent assay (ELISA) kits for detection of antigens of dengue, Chikungunya, West Nile and Sindbis viruses as well as the reverse transcription polymerase chain reaction (RT-PCR) with specific primers and Sanger sequencing.

Results. A total of 102 mosquitoes belonging to three genera, *Culex spp.*, *Culiseta spp.*, *Aedes spp.*, were studied. Mosquitoes of each species or genus were divided into pools, each containing 4–5 mosquitoes. The study of suspensions of only 2 mosquito pools obtained from *Aedes aegypti* and *Aedes albopictus*, starting from the 3rd passage, showed changes in the C6/36 cell monolayer. Starting from the 4th passage, an antigen of Chikungunya virus was detected using ELISA test in the suspension obtained from the *Aedes albopictus* pool. Dengue virus was detected in the 5th passage from the materials obtained from the *Aedes aegypti* pool. Thus, antigens of the Chikungunya and dengue viruses were detected only in 2 of 23 examined pools of mosquitoes of different genera. Materials of the 5th passage were analyzed by RT-PCR with specific primers for dengue and Chikungunya viruses. It was confirmed that the isolate obtained from *Aedes albopictus* mosquitoes contained RNA of the Chikungunya virus and corresponded to the East/Central/South African genotype, while the isolate obtained from *Aedes aegypti* mosquitoes contained RNA of the dengue type 2 virus.

Conclusion. The obtained nucleotide sequences of the Chikungunya virus were deposited in the GenBank international database under accession numbers MN271691 and MN271692.

Keywords: arboviruses; dengue; Chikungunya; mosquitoes; *Aedes spp.*; identification; ELISA; PCR; sequencing.

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Индикация и идентификация вирусов денге и Чикунгунья в комарах рода *Aedes spp.*, отловленных в Центральной Америке

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Целью работы было выделение арбовирусов из комаров различных видов в культуре клеток и их идентификация молекулярными и иммунохимическими методами.

Материалы и методы. Выделение вирусов проводилось на клетках C6/36. Возбудителей идентифицировали с использованием наборов для иммуноферментного анализа (ИФА) для выявления антигенов вирусов денге, Чикунгунья, ВЗН и Синдбис и полимеразной цепной реакции с обратной транскрипцией (ОТ-ПЦР) со специфическими праймерами с последующим секвенированием по Сенгеру.

Результаты. Всего было исследовано 102 комара, относящихся к трем родам: *Culex spp.*, *Culiseta spp.*, *Aedes spp.* Комары каждого вида или рода были разделены на пулы по 4–5 особей. При исследовании суспензий только 2 пулов комаров, полученных от *Aedes aegypti* и *Aedes albopictus*, начиная с 3-го пассажа отмечены изменения монослоя клеток C6/36. В материалах суспензии, полученной из пула *Aedes albopictus*, начиная с 4-го пассажа методом ИФА выявлялся антиген вируса Чикунгунья. В материалах, полученных из пула *Aedes aegypti*, на 5-м пассаже определялся вирус денге. Таким образом, только в 2 из 23 исследованных пулов комаров разных родов определялись антигены вирусов Чикунгунья или денге. Материалы 5-го пассажа были исследованы в ОТ-ПЦР со специфическими праймерами к вирусам денге и Чикунгунья. Подтверждено, что изолят, полученный от комаров *Aedes albopictus*, содержал РНК вируса Чикунгунья и соответствовал Восточному/Центральному/Южно-Африканскому генотипу, а изолят, полученный от комаров *Aedes aegypti*, содержал РНК вируса денге 2-го типа.

Заключение. Полученные нуклеотидные последовательности вируса Чикунгунья были депонированы в международной базе данных GenBank под номерами MN271691 и MN271692.

Ключевые слова: арбовирусы; денге; Чикунгунья; комары; *Aedes spp.*; идентификация; ИФА; ПЦР; секвенирование.

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Introduction

Mosquitoes of different genera transmit a large number of disease-causing viruses, primarily belonging to families *Flaviviridae* (genus *Flavivirus*) and *Togaviridae* (genus *Alphavirus*) [1, 2]. The habitat of *Aedes spp.*, *Culex spp.*, *Culiseta spp.* mosquitoes is not limited to tropical and subtropical countries. These species of mosquitoes are widespread in countries with moderate climate in the southern and northern hemispheres [2–4]. Therefore, there is a high probability that transmitters can interchange, thus causing changes in virus infectivity for humans [5].

Three genotypes have been described for the Chikungunya virus: East/Central/South African, West African and Asian [6]. The improved detection of the Chikungunya virus in collected mosquitoes and in diseased people made it possible to thoroughly study nucleotide sequences of its isolates and changed the beliefs about the genotypes of this pathogen. For example, some researchers speak about genotypes of the Indian Ocean and the Caribbean lineage [6–8]. Only 4 serotypes have been described for the dengue virus [5, 9]. Considering the increasing detectability of viruses transmitted by mosquitoes and the fact that they are pathogenic for humans, development of prevention methods and treatment of diseases caused by alphaviruses and flaviviruses is highly significant. Therefore, the isolation of

viruses directly from the transmitters captured in their natural habitats and the study of the isolated strains are constituent parts of this process.

The purpose of this study was isolation and identification of arboviruses belonging to the *Flavivirus* and *Alphavirus* genera from mosquitoes of *Aedes albopictus*, *Aedes aegypti*, *Culiseta spp.*, *Culex spp.* species.

Materials and methods

Mosquitoes were captured during the dry season in the forested areas of Nicaragua (Tipitapa municipality) having 12.325527N 85.974662W and 12.323326N 85.974275W coordinates. Among the captured mosquitoes there were representatives of *Aedes* genera (*Aedes albopictus*, *Aedes aegypti* species), *Culiseta spp.* and *Culex spp.* genera. After their species had been identified, the mosquitoes were separated into pools of 4–5 insects of the same species. Each pool was homogenized in 0.4 ml of phosphate-buffered saline, pH 7.4, to obtain the suspension.

Isolation of viruses. The obtained mosquito suspension was applied to the monolayer of C6/36 mosquito cells, which was grown in cell culture flasks (Corning, USA) having a 25 cm² surface area. After one-hour adsorption, DMEM cell medium (Chumakov Federal Scientific Center for Research and Development of Immune and Biological Products) was added to the flasks,

together with 2% fetal serum (Gibco). The flasks were placed into an incubator and remained there at 32°C and 5% CO₂. The monolayer was monitored daily under a microscope until a cytopathic effect.

Identification of virus antigens. Reagent kits from Bioservice, namely BioScreen-Dengue (AG), BioScreen-Chikungunya (AG), BioScreen-WNV (AG), BioScreen-Sindbis (AG), were used to detect antigens of Chikungunya, dengue, Sindbis and West Nile in samples of the cell culture fluid. The reaction was performed and the results were recorded in accordance with the instructions to the reagent kits.

Polymerase chain reaction (PCR). The AmpliSens[®] Magno-Sorb (InterLabService) kit was used to isolate RNA from 100 µl of the virus-containing culture fluid in accordance with the manufacturer's protocol.

The reverse primers pE2CHVrev1, pE2CHVrev2, pE1CHVrev1, pE1CHVrev2, pNS1CHVrev1, pNS1CHVrev2, pNS1CHVrev3 for the Chikungunya virus (**Table 1**), pDV2rt (5'-CAGCCATGGCAGCGGTAGGTC-3') primers for the dengue virus, and the reagent kit for the reverse transcription (RT) (Syntol) were used to perform the RT reaction on the virus RNA matrix and to receive cDNA. During the first stage, 2 µl of the reverse primer (10 pmol/µl) were mixed with 6 µl of the isolated RNA, and then the mixture was heated during 5 minutes at 95°C. Then the tubes were cooled down during 2 minutes at the indoor temperature and were filled with another 22 µl of the mixture for RT (9 µl of deionized water, 12 µl of 2.5-fold buffer for RT, 2.5 µl of MMLV- reverse transcriptase and were incubated during 30 minutes at 42°C. The mixture was

heated during 5 minutes at 95°C to inactive the reverse transcriptase.

Oligonucleotides shown in Table 1 were used for obtaining *E1*, *E2* and *NS1* gene fragments of the Chikungunya virus and their sequencing.

The PCR on the cDNA obtained after RT was performed by using the following combinations of primers for the Chikungunya virus:

- gene *E1* — pE1CHVfor1 and pE1CHVrev1, pE1CHVfor2 and pE1CHVrev2;
- gene *E2* — pE2CHVfor1 and pE2CHVrev1, pE2CHVfor2 and pE2CHVrev2;
- gene *NS1* — pNS1CHVfor1 and pNS1CHVrev1, pNS1CHVfor2 and pNS1CHVrev2, pNS1CHVfor3 and pNS1CHVrev3.

For the dengue virus, the PCR was performed as described previously [10]: pDV2for (5'-CCAAA-CAACCCGCCACTCTAAGG-3') and pDV2rev (5'-GTTATCACGACAGTGTATTCCA-3'). The PCR mixture was received by mixing of 1 µl of the forward and 1 µl of the reverse primer at 10 pmol/µl, 10 µl of the multipurpose 2.5-fold reaction mixture for PCR (Syntol), 8 µl of deionized water, and 5 µl cDNA added later. The amplification was conducted by using the XP Cyclor instrument (Hangzhou Bioer Technology) according to the following protocol: 95°C — 1 min 30 sec; 30 cycles: 95°C — 20 sec, 55°C — 15 sec, 72°C — 30 sec; the final elongation at 72°C — 10 min. The protocol for amplification of the dengue virus: 95°C — 1 min 30 sec; 40 cycles: 95°C — 20 sec, 57°C — 15 sec, 72°C — 30 sec; the final elongation at 72°C — 10 min.

Table 1. Primers used for RT, PCR and sequencing of the Chikungunya virus

Ref No. of primers	Primer index	Protein gene	Sequence of the primer	Coordinates	Size of the PCR-product, b.p.
1	pE1CHVfor1	<i>E1</i>	5'-GAACTGACACCAGGAGCTACCGTCC-3'	9745–10600	856
	pE1CHVrev1		5'-CGCCAAATTGCTCTGGTCTTCCTG-3'		
2	pE1CHVfor2		5'-AACATGGACTACCCGCCCTT-3'	10552–11313	762
	pE1CHVrev2		5'-GTGCCTGCTRAACGACACGC-3'		
1	pE2CHVfor1	<i>E2</i>	5'-TTCAATGTCTATAAAGCCACAAGACC-3'	8560–9240	681
	pE2CHVrev1		5'-GTGATTGGTGACCGCGGCATG-3'		
2	pE2CHVfor2		5'-GYCAGACGGTGCGGTACAAGTG-3'	9125–9794	670
	pE2CHVrev2		5'-GCAGCATATTAGGCTAAGCAGGAAAG-3'		
1	pNS1CHVfor1	<i>NS1</i>	5'-ATGGATYCTGTGTACGTGGAYATAGAC-3'	80–572	493
	pNS1CHVrev1		5'-GGTGRATATAGCGACGTGGGTGC-3'		
2	pNS1CHVfor2		5'-CATGTAGACAGAGAGCAGACGTCGC-3'	498–1139	642
	pNS1CHVrev2		5'-CCTCCGGCGTGACTTCTGTAGC-3'		
3	pNS1CHVfor3		5'-CGTGCCGGCGACCATTGTG-3'	1078–1710	633
	pNS1CHVrev3		5'-GCKCCTCTMGAGTCTCTATTATCC-3'		

The measuring of the minimum infection rate (MIR) of the studied mosquitoes was conducted as described previously [11, 12].

Results

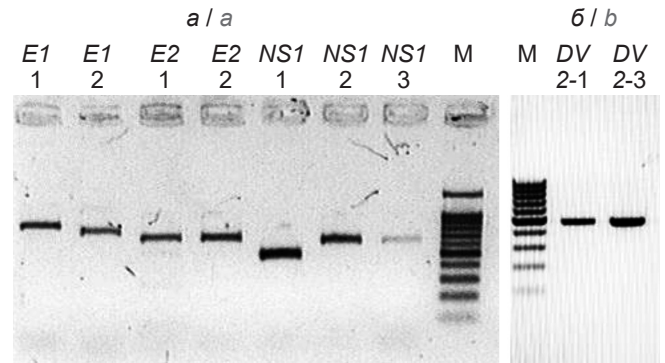
A total of 102 mosquitoes of three genera were studied (Table 2). The largest number of mosquitoes belonged to the *Aedes* genus represented by *Aedes aegypti* and *Aedes albopictus* species. Each sample of suspension went through 4 successive “blind” passages on C6/36 cells. The condition of the cell monolayer was monitored under the microscope during 5 days after the passage.

As a result, it was confirmed that the isolate obtained from *Aedes albopictus* mosquitoes contained the RNA of the Chikungunya virus and did not contain the RNA of the dengue virus. The isolate obtained from *Aedes aegypti* mosquitoes contained the RNA of the dengue virus and did not contain the RNA of the Chikungunya virus. Based on the results of sequencing, the isolate of the dengue virus was classified as type 2. The PCR products of *E1*, *E2* and *NS1* genes, which were obtained by the amplification of the isolate of the Chikungunya virus, were also sequenced.

The obtained sequences are deposited in the GenBank under numbers MN271691 and MN271692. The BLAST computer program was used for classification of the nucleotide sequences of the isolate of the Chikungunya virus, which were classified as the East/Central/South African genotype.

Changes in the cell monolayer were detected only in 2 pools of *Aedes aegypti* and *Aedes albopictus* mosquitoes, starting from the 3rd passage. On the 4th passage of the *Aedes aegypti* pool, the cytopathic effect was observed on the 4th day, while the *Aedes albopictus* pool demonstrated it on the 3rd day. After the additional 5th passage on the C6/36 cells, the materials of the above pools were examined with ELISA (reagent kits from Bioservice) for presence of antigens of dengue, Chikungunya, West Nile and Sindbis viruses.

In the *Aedes aegypti* and *Aedes albopictus* pools, in the materials of all the examined passages, no antigens of West Nile and Sindbis viruses were found (Table 2). At the same time, starting from the 4th passage,



The electrophoresis of gene fragments of Chikungunya (a) and dengue (b) viruses in 1% agarose gel.

a — PCR products of *E1*, *E2* and *NS1* gene fragments of the Chikungunya virus. 1–3 — the reference numbers of primers (Table 1);

b — PCR products of the gene fragment of the surface protein of the dengue virus, which was obtained on the 4th (DV2-1) and 5th (DV2-3) passages. M 100 bp — the DNA molecular weight marker, 100 bp.

the antigen of the Chikungunya virus was detected in the materials of the suspension obtained from the *Aedes albopictus* pool. Starting from the 5th passage, the dengue virus was detected in the materials from the *Aedes aegypti* pool (Figure). Thus, antigens of Chikungunya or dengue viruses were detected only in 2 out of 25 examined pools of mosquitoes of different genera.

The minimum infection rate (MIR) for *Aedes aegypti* was 0.0357, for *Aedes albopictus* — 0.0270. Thus, the pools containing antigens of Chikungunya and dengue viruses had a cytopathic effect on Cy6/36 on the 4th and 5th successive passages. The material of the 5th passage of both pools of mosquitoes was analyzed by using RT-PCR with specific primers for dengue and Chikungunya viruses.

Discussion

In countries of Central America, the transmission of dengue and Chikungunya viruses is caused by mosquitoes of the *Aedes* species. Insects are generally collected in areas of human residence [2–5, 9, 12]. In our study, mosquitoes of 3 species were captured outside human settlements, in the forested area. When examining whether mosquitoes of these species are infected with a virus, the number of mosquitoes in the studied

Table 2. Mosquito genus and total number of mosquitoes; number of studied pools

Mosquito genus	Number of mosquitoes	Number of pools	ELISA		PCR	
			dengue	Chikungunya	dengue	Chikungunya
<i>Culex spp.</i>	16	4	0/4	0/4	0/4	0/4
<i>Culiseta spp.</i>	21	5	0/5	0/5	0/5	0/5
<i>Aedes spp.</i>	65	16	1/16	1/16	1/16	1/16
<i>Aedes aegypti</i>	28	7	1/7	0/7	1/7	0/7
<i>Aedes albopictus</i>	37	9	0/9	1/9	0/9	1/9

pool is of high importance. The optimum size of the pool is a 4 mosquito pool [2, 11, 12]. PCR is used for detection of viruses in mosquito pools; if the result is positive, the obtained amplicon is sequenced. The published articles have no data about using of ELISA.

As the size of the primary suspension of mosquitoes in our study was limited, detection of viruses after “blind” successive passages on C6/36 cells is completely justified. Using ELISA and RT-PCR for detection of viruses makes it possible to perform detection by employing two independent methods.

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