

Existence of Hepatitis C Virus in *Culex quinquefasciatus* after Ingestion of Infected Blood: Experimental Approach to Evaluating Transmission by Mosquitoes

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Received 2 March 2001/Returned for modification 4 May 2001/Accepted 28 June 2001

We used PCR to detect hepatitis C virus (HCV) RNA among supernatants of ground *Culex quinquefasciatus* mosquitoes that (i) had been fed HCV-positive blood, (ii) had been intrathoracically inoculated with HCV-positive blood, or (iii) were from homes of hepatitis C patients. HCV RNA was detectable under all three conditions, but it did not replicate in mosquitoes and was not detectably transmitted during feeding.

Hepatitis C virus (HCV) is an RNA virus causing sporadic non-A, non-B hepatitis. Other HCV transmission routes may exist in addition to the obvious parenteral routes of transmission. HCV, pestivirus, and flavivirus are of the family *Flaviviridae*, containing over 60 arthropod-borne viruses. Many flaviviruses (causing yellow fever, Japanese encephalitis, dengue) are transmitted by mosquitoes. HCV has been proven to replicate in mosquito cells (R. Germi, J. M. Crance, and D. Garin, Abstr. 100th Gen. Meet. Am. Soc. Microbiol., abstr. A-16790, 2000). Therefore, we investigated possible mosquito-borne HCV replication and transmission. Taiwan is subtropical. *Culex quinquefasciatus* is common in tropical and subtropical areas of Asia, Africa, and America. Because our preliminary work detected HCV RNA in *C. quinquefasciatus* more than in *Aedes aegypti* or *Aedes albopictus*, *C. quinquefasciatus* was studied and is described herein.

A continuous laboratory colony of *C. quinquefasciatus* (Jin-Men strain) was maintained. Four-day-old females, deprived of 10% sucrose solution meal for 24 h, were tested. The feeding apparatus was from Wade (6). HCV-positive whole blood, confirmed through nested PCR, was stored at 4°C and heated to 37°C for feedings.

Twenty 4-day-old female mosquitoes were ground separately, immediately (day zero) after feeding on HCV-positive blood. Supernatant solutions were prepared. An additional 260 mosquitoes were similarly tested from days 1 to 30 after feeding. Another group of mosquitoes ($n = 90$) was infected by intrathoracic inoculation (0.96 μ l) using pulled disposable pipette needles (4). Supernatants were prepared and tested as described above for a similar number of days. A total of 49 female *C. quinquefasciatus* mosquitoes, collected at three homes of patients with chronic HCV infection, were tested as described above. Of these 49 mosquitoes, 27 had recently fed.

Supernatants of all mosquitoes were tested for both positive- and negative-stranded HCV RNA by PCR.

Oral transmission was tested by inserting mosquito proboscises into capillary pipettes containing 3.5 μ l of a solution (10% fetal bovine serum, 10% sucrose) for 60 to 90 min (5). Pipette contents were tested for HCV RNA. Groups of 15 mosquitoes were tested at 9, 11, 17, 21, and 25 days (75 mosquitoes in total) after HCV-positive-blood feeding, for biological oral transmission. Groups of 30 mosquitoes were tested, immediately after and 10 and 30 min after HCV-positive-blood feeding, for mechanical oral transmission via small quantities of blood on mosquito proboscises.

The mosquito supernatant RNA extraction was performed using a modified single-step RNA isolation method. Positive-stranded HCV RNA cDNA was synthesized at 37°C, for 3 h, with antisense primer 5'-AACACTACTCGGCTAGCAGT-3' and moloney murine leukemia virus reverse transcriptase. Negative-stranded HCV RNA cDNA was synthesized similarly using sense primer 5'-ACTCCACCATAGATCATCCC-3'. Products were boiled to inactivate reverse transcriptase activity and then treated with RNase A to digest all RNA molecules and avoid possible amplification of positive-stranded HCV RNA (1). Appropriate controls were included with each assay. Nested PCR amplification from the 5' noncoding region was performed with corresponding primer pairs (outer primers: 5'-ACTCCACCATAGATCATCCC-3', nucleotides [nt] 23 to 42, and 5'-AACACTACTCGGCTAGCAGT-3', nt 264 to 245; inner primers: 5'-TTCACGCAGAAAGCGTCTAG-3', nt 62 to 81, and 5'-GTTGATCCAAGAAAGGACCC-3', nt 206 to 187). Aliquots (15 μ l) of the PCR products were analyzed by agarose gel electrophoresis. The detection sensitivity of the nested PCR was about 10 to 100 HCV genome equivalents.

To confirm PCR products as specific and full-length HCV genomes, extracted RNA was tested with four other primer sets specific for the core (outer primers: 5'-CGCGCGACTA GGAAGACTTC-3', nt 480 to 499, and 5'-ATGTACCCCAT GAGGTCGGC-3', nt 751 to 732; inner primers: 5'-AGGAA GACTTCCGAGCGGTC-3', nt 489 to 508, and 5'-GAGCCA TCCTGCCACCCCA-3', nt 632 to 613), E1/E2 (outer

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5' NCR												
Patient Mosquito	93	AGTATGAGTG	TCGTGCAGCC	TCCAGGACCC	CCCCTCCCGG	GAGAGCCATA	GTGGTCTGCG	GAACCCGGTGA	GTACACCAGGA	ATTGCCAGGA	CGACCCGGGTC	192
Taiwanese strain		-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	
Patient Mosquito	193	CTTTCTTGA	TCAACA									208
Taiwanese strain		-----	-----C									
Core region												
Patient Mosquito	514	CTCGTGAAG	GCGACAACCT	ATCCCCAAGG	CTCGCCGACC	CGAGGGCAGG	ACCTGGGCTC	AGCCCCGGTA	TCCTTGCCCC	CTCTATGGCA	ACGAGGGCAT	613
Taiwanese strain		-----	-----	-----	-----AG--	-----	G-----	-----	C-----	--T-----	-T-----T-	
Patient Mosquito	614	GGGGTGGCA	GGATGGCT									631
Taiwanese strain		-----	-----									
E1/E2 region												
Patient Mosquito	1375	TGGACGTGT	GACAGGGGCC	CATTGGGGAG	TCCTGGCGGG	CCTTGCCTAC	TATTCATGG	TGGCAAAGT	GGCTAAGGTT	CTGATTGTGA	TGCTGCTTTT	1474
Taiwanese strain		---TA---	-GGG----	-C-----	-----	-----	-----	--GG----	-----	--A-----	---A----	
Patient Mosquito	1475	TGCCGGCGTT	GATGGGCACA	CCCACACAAT	AGGAGCGGTG	GAGGGCCGCC	ACACCTCCGG	CTTTACGGGT	CTCTTTTCAT	CTGGGTCTGC	ACAGAAAATC	1574
Taiwanese strain		-----	--C--AGT-	-AT--GTGC	---G-ACA	-T--C---A	C---CA-A-	TC-CG--TC-	-----A--C	AA--G-GT-	C-----	
Patient Mosquito	1575	CAACTTG										1881
Taiwanese strain		--G--A										
NS3 region												
Patient Mosquito	4756	TTGAAACGAC	GACCGTGCCC	CAAGACGCGG	TGTCGCGCTC	GCAGCGGCGA	GGTAGGACTG	GCAGGGGTAG	GGTAGGCATC	TACAGTTTGG	TGACTCCAGG	4855
Taiwanese strain		---G----	---A----	-----	---A----	-----	-C-----A	-T-----C--	-AG-----	-----C-	-----	
Patient Mosquito	4856	AGAACGGCCC	TCAGGCATGT	TCGACTCTTC	GGTCTGTGT	GAGTGTCTATG	ACGCGGGCTG	TGCGTGGTAC	GAGCTTACGC	CCGCTGAGAC	CACGGTTAGG	4955
Taiwanese strain		-----	--G-----	---T-----	-----C	-----	-----	---T-----	-----C-A-	---C-----	-T-----	
Patient Mosquito	4956	TTGCGGGCGT	ACCTAAATAC	ACCAGGGTTG	CCCCTTTGCC	AGGACCATTT	GGAGTTCTGG	GAGGGTGTCT	TCACAGGCCT	TACCCACATA	GATGCCCACT	5055
Taiwanese strain		-----T-	-----	-----	-----	-----C-	-----	---A-C---	-----	-----	-----T-	
Patient Mosquito	5056	TCTTGTCCCA	GACTAAGCAG	GCAGGAGACA	ACTTCCCTTA	CCTGGTGSCA	TACCAGGCCA	CAGTGTGCGC	CAGGGCTCAG	GCTCCACCTC	CATCATGGGA	5155
Taiwanese strain		-----	-----	-----	-----	---A---	---A--A-	-G---T--	-----	-----G--	-----	
Patient Mosquito	5156	TCAATGTGG	AAGTGTCTCA	TACGGCTAAA	ACCTACATTG	CAGGGGCCAA	CACCCCTGCT	GTACAGGCTA	GGAGCCGTCC	AAAATGAGAT	CACCC	5250
Taiwanese strain		C-----	-----	C-----	---C---C-A	-----	-----T-	-----A-G	-----	-----G-	-----	
NS5 region												
Patient Mosquito	6899	TAAGCGAAGG	CTGGCCAGGG	GGTCTCCCCC	CTCCTTGCC	AGTTCCTCAG	CTAGCCAGTT	GTCTGCGCCT	TCTTTGAAGG	CGACATGCAC	TACACATCAC	6998
Taiwanese strain		-----T--	-----	-----	-----	--C--T--	-----	-----A-T-	---C---A-	-G-G-----	C--C-G--T	
Patient Mosquito	6999	GACTCCCGGG	ATGCCGATCT	CATCGAGGCC	AACCTCTGT	GGCGGAGGA	GATGGCGGGG	AACATCACCC	GTGTGGAGTC	AGAGAACAAG	GTAGTAATTT	7098
Taiwanese strain		AC-C---A-	-C--T--C--	---T-----	-----	-----	-----A-	-----	-C-----	-----T--	-----CC	
Patient Mosquito	7099	TGGACTCTTT	CGACCCGCTT	CGAGCCGAAG	AGGATGAGAG	GGAATATCC	GTTGCGGCGG	AGATCCTGCG	GACATCCAGG	AGGTACCCCC	CAGCGATGCC	7198
Taiwanese strain		-----	-----C	-----G-	---C-----	---G----	-----C-	-----	--A-----	-A--T---T-	-----T---	
Patient Mosquito	7199	CATATGGGCA	CGGCCGGATT	ACAACCCTCC	GCTGATAGAG	TCCTGGAAGA	ACCCGGACTA	CGTCCCTCCT	GTGGTACACG	GGTGCCCACT	GCCGCTGTC	7298
Taiwanese strain		-G-----	---C-----	-----	AT--C-----	C-----AG	-----	-----A	-----CT-	---A-----	-----	
Patient Mosquito	7299	AAGGCCCCCC	CAGTACC									7315
Taiwanese strain		-----T-	--A----									

FIG. 1. Nucleotide sequence alignment of the 5' noncoding region, core, E1/E2, NS3, and NS5 fragments of HCV from a patient and mosquitoes. The sequences are compared with the Taiwanese strain of HCV, as published by Chen et al. (2) (EMBL/GenBank M84754). The nucleotide numbering system is according to that of GenBank sequence number M84754.

primers: 5'-TGGCTTGGGACATGATGATG-3', nt 1294 to 1313, and 5'-TCACAACGCTCTCCTCGGGT-3', nt 2299 to 2280; inner primers: 5'-TGCTCCGGATCCCTCAAGC-3', nt 1351 to 1369, and 5'-TGATGTGCCAGCTGCCATTG-3', nt 1608 to 1589), NS3 (outer primers: 5'-GTAACACGTGTGT CAC-3', nt 4702 to 4717, and 5'-GCATGCCATGATGTAT-3', nt 5285 to 5270; inner primers: 5'-GACAGTCGACTTCA GCTT-3', nt 4721 to 4738, and 5'-TGGTTATGGGGTGCCT GA-3', nt 5268 to 5251) and NS5 (outer primers: 5'-CATGT AAACCTCTCCTACGG-3', nt 6748 to 6767, and 5'-TAGCA AGCTCTGCCAAAGCA-3', nt 7386 to 7367; inner primers: 5'-AATACGTGGTTGGGTCACAG-3', nt 6798 to 6817, and

5'-CCTCTTTCTCCGTGGAGGTG-3', nt 7334 to 7315) HCV regions. We tested two mosquitoes with each primer set, 1 and 2 days after HCV-positive-blood feeding with the blood used in the laboratory feeding, a mosquito trapped in a hepatitis C patient's home, and the patient's serum. PCR products were purified and sequenced directly using sense and nested primers with a cycle-sequencing protocol that incorporated fluorescent dye terminators via automatic sequencer analysis and PILEUP software.

As measured in a branched DNA assay, the virus titer of the whole blood used for feeding mosquitoes was 7.908 Meq/ml. The HCV RNA detection rates in the mosquitoes were 100%

(40 of 40) and 13% (32 of 240) within 1 day and 2 to 30 days after HCV-positive-blood feeding, respectively, compared to 70% (14 of 20) and 11% (8 of 70) after intrathoracic inoculation. HCV RNA could be detected in some mosquitoes up to 25 days after feeding. For mosquitoes trapped in hepatitis C patients' homes, the detection rate was 11% (3 of 27). Negative-stranded HCV RNA was undetectable at any time in any case, indicating a lack of HCV RNA replication. Tests of biological and mechanical HCV transmission yielded negative results.

In sequence analysis and comparison of PCR products for five HCV regions, all PCR products were found to be HCV positive except for one day-2 mosquito, which tested HCV negative for the NS5 region. All HCV isolated from mosquitoes was genomically identical to HCV in the source blood. Homology with the Taiwanese strain (2) in the five regions was 99.1, 94.1, 76.8, 92.3, and 88.0%, respectively (Fig. 1), confirming that the PCR products were specific and represented the full-length HCV genome.

This study showed HCV existence in the majority of *C. quinquefasciatus* organisms studied within 1 day after HCV-positive-blood feeding and intrathoracic inoculation, with the latter exposure route included to avoid possible midgut HCV barriers. The HCV detection rate declined rapidly, consistent with findings in mosquitoes from hepatitis C patients' homes and other reports concerning hepatitis B virus (HBV) and *C. quinquefasciatus* (3), although HCV could be detected in some mosquitoes for up to 25 days. Furthermore, we showed no HCV replication in these mosquitoes, even when the midgut was bypassed by employing intrathoracic inoculation. This ev-

idence strongly weighs against mosquitoes being reservoirs of HCV.

Arthropod vectors have been suspected in mechanical HBV transmission (7). However, our tests of biological and mechanical HCV transmission by *C. quinquefasciatus* showed negative results. Low HCV RNA titers in patient sera and different species tropisms for HCV RNA replication are probably the reasons why mechanical and biological HCV transmission does not occur in mosquitoes. It seems *C. quinquefasciatus* is not an HCV risk factor.

This work was supported by grants NSC85-2331-B006-024-MH and NSC86-2315-B006-001-MH from the National Science Council.

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