Role of *Culex* mosquito in the transmission of hepatitis C virus: an experimental study in Iraq

Hashim R. Tarish[1], Ghanim A. Al-Mola[2], Karar M. Abdulsada[1], Rafah Hady Lateef[2], Ghazi R Auda[1]*

[1]Department of Microbiology, College of Medicine, University of Kufa, Iraq
[2]Department of Biology, College of Science for Women, Babylon University, Babylon-Iraq
*Correspondence Author: Dr. Ghazi R Auda; (audagr@googlemail.com)

Abstract

The study involved evaluation of the role of *Culex* mosquitoes in the transmission of hepatitis C virus (HCV), after feeding the insects on infected blood with a viral load of 1.3 x 10^5 IU/ml. Viral nucleic acid was detectable in the insects by RT-PCR assay for up to thirteen days post feeding with the HCV containing blood meal. These insects were left to feed on viral free blood later on this blood was tested by RT-PCR assay and gave negative result. Testing of HCV-free blood that was used to feed insects, already fed on HCV infected blood, gave negative results for HCV virus genome. Viral stability criteria were investigated on infected blood samples with known viral load of (1.3 x 10^5 IU/ml). Viral nucleic acid was detectable by RT-PCR test for up to 9 days when whole blood stored at 4ºC; for 3 days when whole blood stored at the room temperature and for 36 hours when dried blood stored at room temperature. The estimation of some viral stability criteria by RT-PCR analysis was first done in our country.

Key words: *Culex* mosquito, hepatitis C virus, RT-PCR

{Citation: Hashim R. Tarish, Ghanim A. Al-Mola, Karar M. Abdulsada, Rafah Hady Lateef, Ghazi R Auda. Role of *Culex* mosquito in the transmission of hepatitis C virus: an experimental study in Iraq. American Journal of Research Communication, 2014, 2(10): 176-185} www.usa-journals.com, ISSN: 2325-4076.

Introduction

Hepatitis C virus infection is one of the major public health problem in both developed and developing countries since discovering at 1989 (1, 2, 3).

It is estimated that HCV infect 200 million peoples (3%) of the world's population and there are at least 21.3 million HCV carriers in the Eastern Mediterranean countries (4). The infection acquired mainly through parenteral route (5), and also perinatally (6) but (30-40%) of infected cases are without identifiable route (7, 8). Many investigators have suggested that the mechanical transmission of HCV by mosquitoes is plausible. (9, 10, 11). Corroborative

data were obtained and indicate the existence and survival of hepatitis C virus in the mosquitoes for many days (12, 13, 14).

The aim of this study is to evaluate the role of mosquitoes in the transmission of HCV.

Materials and Methods

Study design: Mosquitoes of *Culex* spp. were experimentally fed with infected blood to evaluate the existence and fate of the virus inside mosquitoes, besides the study of some viral stability criteria.

The Mosquitoes: The larvae and pupae were collected from a site of dirty water drainage at Al-Qizwiniya campus (the Colleges of Science and Agriculture, Kufa University) by use of white tulle dipper with long arm. In order to see the larvae and pupae that put in plastic basins which contain chlorine free water (tap water that left at least one day before the collection). Larvae and pupae then transmitted to the insect laboratory at the College of Agriculture, University of Kufa and transferred by use of large opening plastic dripper to plastic containers (20x15x8 cm) with 2 liters of chlorine free water and fed by 2gm of the mice ration, after that they placed inside four breeding cages (70x60x60 cm) that made from wood and covered by white tulle then equipped by plastic Petri dishes containing pieces of cotton soaked with 10% sucrose solution for feeding of newly emerged mosquitoes that occurred after 2-4 days.

On day three after emerging from pupae, adult female mosquitoes were allowed to feed on fresh pigeon blood by using of live pigeon as described elsewhere (15). Breeding cages are equipped with small plastic basins (15x10x7 cm) containing tap water necessary for egg laying, which appeared as egg rafts 3-4 days after blood feeding. Egg rafts were transferred, using a small brush, to other plastic basins contain tap water containing mice ration. Water was changed every 4 days to maintain larvae activity and to prohibit water spoiling. Eggs normally hatched 3-5 days after being laid. This is followed by development of larvae and pupae (16).

The above steps were repeated three consecutive times to obtain a colony of fair number of *Culex* mosquitoes. Samples from larvae-4 and adults of the third generation have been identified as *Culex* spp. by the Entomologist Dr. Mushtaq Al-Ghazali of the College of Agriculture, Kufa University and by the specialist the assist prof. Dr. Ghyda A. Al-Gubory department of parasitology, College of Veterinary Medicine, Al-Qadisya University. After identification as a *Culex spp.*, the colony was considered as ready to use for the experimental study.

Measurement of viral blood load

In order to detect the exact quantities of HCV load in blood samples, the probe hydrolysis technique of Taq-man probe has been adopted as it is considered as an appropriate procedure for viral quantification by the use of Real Time PCR.

The standard curve in this method was first constructed from cDNA of known concentration that was then used as a reference standard for extrapolating of target RNA concentration in tested samples. The method was found to be reproducible and having a high dynamic range of detection (Figure 1).

Estimation of the Role of *Culex* Mosquitoes in the Transmission of HCV Infection

Feeding of Mosquitoes with Infected Blood

Two of modified, home-made, *in vitro* blood feeder of mosquitoes were prepared by inducing circular hole with diameter of 12 cm in the base of each breeding cage (70x60x60 cm) using a special saw, a glass Petri dish was fixed in the hole with silicon glue.

A water bath containing a glass jar fully filled with water, than the cages laid on the water baths in way that the glass Petri dish was fully touched the water surface of the glass jar.

Approximately 300 of female mosquitoes from the third generation (2-3 days after emergence of adult mosquitoes) were starved for 24 hours then transferred to one of these cages by use of modified sucking tube (aspirator). The temperature of the *Culex* mosquitoes breeding was 22-28˚C, a sterile plastic Petri dish containing sterile piece of cotton soaked in 5 ml of fresh infected blood of viral titer $1.3x10^5$ IU/ml was covered by thin layer of Para film. The glass Petri dish was then placed in the cage with a thermostat attached to the Parafilm as shown in the figure (Figure 2). When the temperature in the water bath reach 45˚C the temperature in the thermostat reach and stabilized at 37˚C, at this time the Para film was removed and the cage covered with black cloth and the room was darkened for 30 minutes. Samples of mosquitoes aspirated and visually inspected for the presence of blood meal, most of which were engorged with blood. Ten of these mosquitoes collected at this time, then all engorged mosquitoes transferred to another cage and sucrose was returned to the mosquitoes.

Ten of the mosquitoes collected at each time in following periods after feeding: Zero time (directly after feeding), 6 hours, 12 hours, 24hours, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15 days. The collected mosquitoes have been sunk in 70 % ethanol and left for 1 minute to dry inside sterile Petri dishes in biological cabinet and placed in safe-lock 0.5 ml sterile Eppendorf tubes containing few drops of sterile phosphate buffer saline and stored at -20˚C until use (17, 18).

Evaluation the Infectivity Through Mouth Part

From day 1 to day 7 after feeding with HCV infected blood, a plastic sterile Petri dish containing 10 ml of fresh HCV-free blood covered with a thin layer of Para film painted with thin layer of blood and placed in the same cage for *in vitro* feeding of mosquitoes and about 100 of male mosquitoes with sucrose plates were added to mimic mosquito nature. A daily collection of 100 µl of this blood was performed from day 2 to day 8 and put into sterile 0.5 ml Eppendorf tubes and stored at -20˚C until use for searching of the virus by reverse transcription PCR.

Estimation of the Viral Nucleic Acid Stability

From a patient with viral titer $3.1x10^5$ IU/ml of blood, 10 ml of blood was taken aseptically and divided into two equal volumes. Five ml of the blood divided into two aliquot

each 2.5 ml in two heparin tubes, one tube stored at 4˚C where as the another tube was stored at room temperature (22-28)˚C. From each tube, a 100 µl of blood was aspirated and placed in 0.5 ml sterile Eppendorf conical flip-cap tubes and stored at -20˚C simultaneously with the times of mosquito sampling at: 6 hours, 12 hours,24 hours and after 2, 3, 4, 5, 6, 7, 8, 9 and 10 days, then submitted to reverse transcription PCR analysis.

The other (non heparinized) 5ml of the patient blood was placed into a 14 cm diameter sterile plastic Petri dish and incubated at 37˚C for 4 hours alone with open cap inside incubator which previously sterilized by 70% ethanol, then the dried blood stored at room temperature. About 100 mg of the dried blood was taken aseptically by sterile blade at following times after dryness: 0, 6, 12, 24, 36, 48, 60, 72, 84 and 96 hours, and put in sterile 0.5 ml Eppendorf tubes each contains 100 µl of nuclease free water mixed and stored at -20˚C until use for reverse transcription PCR analysis.

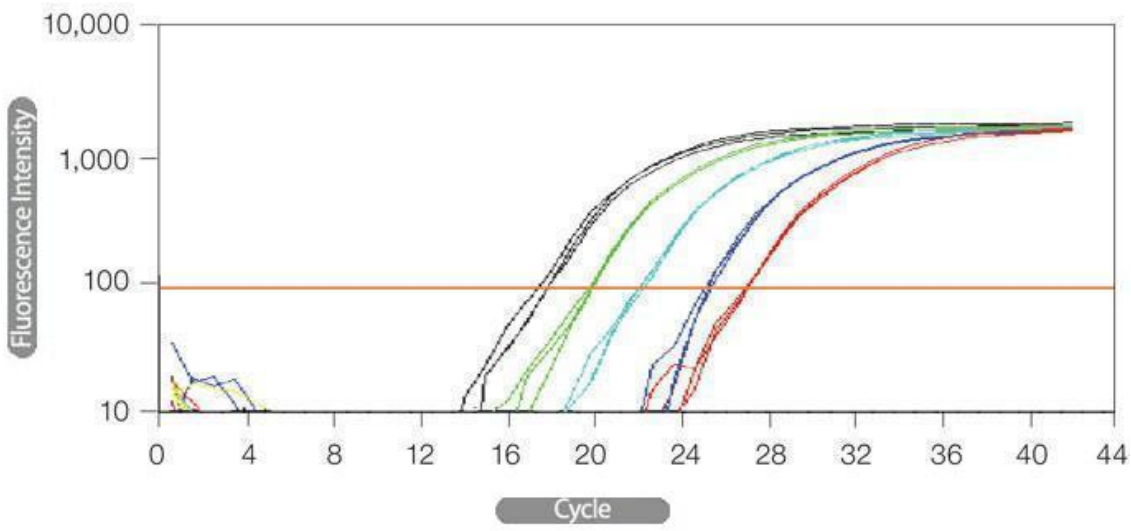

Figure 1: Ten-folded serial dilution of the standard positive control sample for obtaining of the standard curve.

Results

Experimental Study on *Culex* Mosquitoes

Females of *Culex* mosquitoes that *in vitro* fed on the HCV infected blood from patient with viral genotype four and concentration of 1.3 x 10^5 IU/ ml were subjected to RT-PCR analysis searching presence the viral nucleic acid inside these insects, the following results were proved from each ten of mosquitoes that collected at different times after feeding. All mosquitoes that are collected at time zero post feeding, and 6 hours post feeding had shown positive results for HCV when RT-PCR analysis was used. Viral nucleic acid had been detected in 8 out of 10 mosquitoes that were tested 12 hours post feeding. Nine out of ten

mosquitoes which were tested at the times 1 and 2 days post feeding were found as having HCV nucleic acid, 2 out of 10 (20%) mosquitoes were still harboring the virus until day thirteen post feeding with HCV containing blood. On the other hand, HCV nucleic acid was not found in mosquitoes that were tested at day 14 and day 15 after feeding with HCV containing blood (Figure 3).

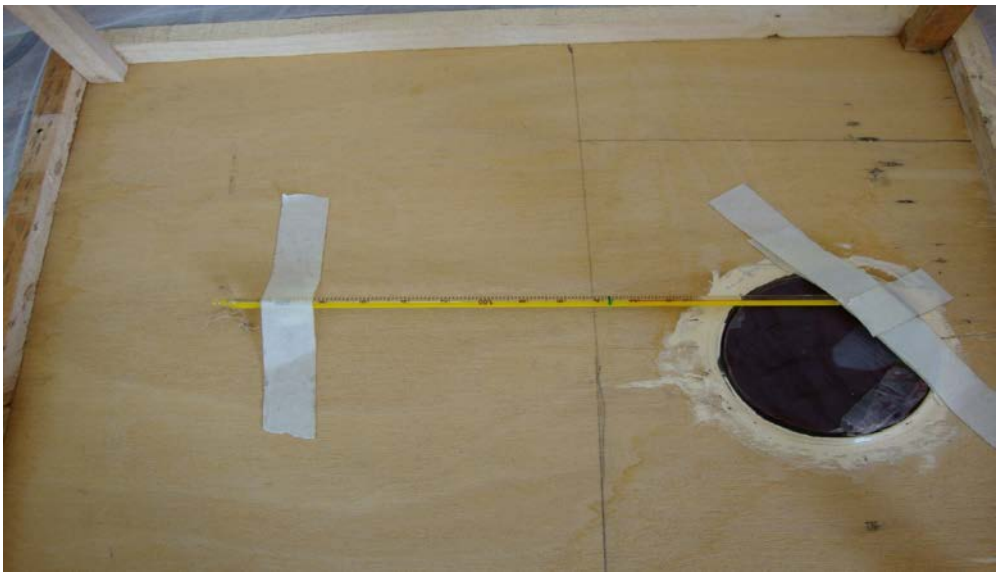

Figure (2): Petri Dish Contain Cotton Soaked with Blood Covered with Parafilm, to it a Thermostat was Attached.

Figure (3): Mosquitoes During *in vitro* Feeding.

The study results also revealed that at the days 9 and 10 after *in vitro* feeding, there was an unexpected increase in the number of mosquitoes that were containing viral genome (6 at each day) followed by a decline in the detection of viral genome from day 11 onward until it became absent on day 13 onward (Table-1).

Estimation of the Infectivity Through Mouth Parts of Mosquitoes

A group of females of *Culex* mosquitoes previously fed on infected blood were left from day 1 to day 7 to feed on uninfected blood through Parafilm. That was intended to check for the possibility of transfer of virus, through saliva, from the mosquitoes to the uninfected blood used for the subsequent feeding. Samples from the blood used for the subsequent feeding were collected daily from day 2 to 8 after initial feeding on infected blood. No HCV genomic RNA was detected using RT-PCR analysis.

Table (1): Number of female mosquitoes containing HCV RNA

Time after feeding (days)	Number of mosquitoes taken	Number of mosquitoes positive for HCV	% of mosquitoes positive for HCV	% of mosquitoes negative for HCV
0	10	10	100	0
0.25	10	10	100	0
0.5	10	8	80	20
1	10	9	90	10
2	10	9	90	10
3	10	6	60	40
4	10	4	40	60
5	10	3	30	70
6	10	5	50	50
7	10	3	30	70
8	10	2	20	80
9	10	6	60	40
10	10	6	60	40
11	10	3	30	70
12	10	2	20	80
13	10	2	2	80
14	10	0	0	100
15	10	0	0	100

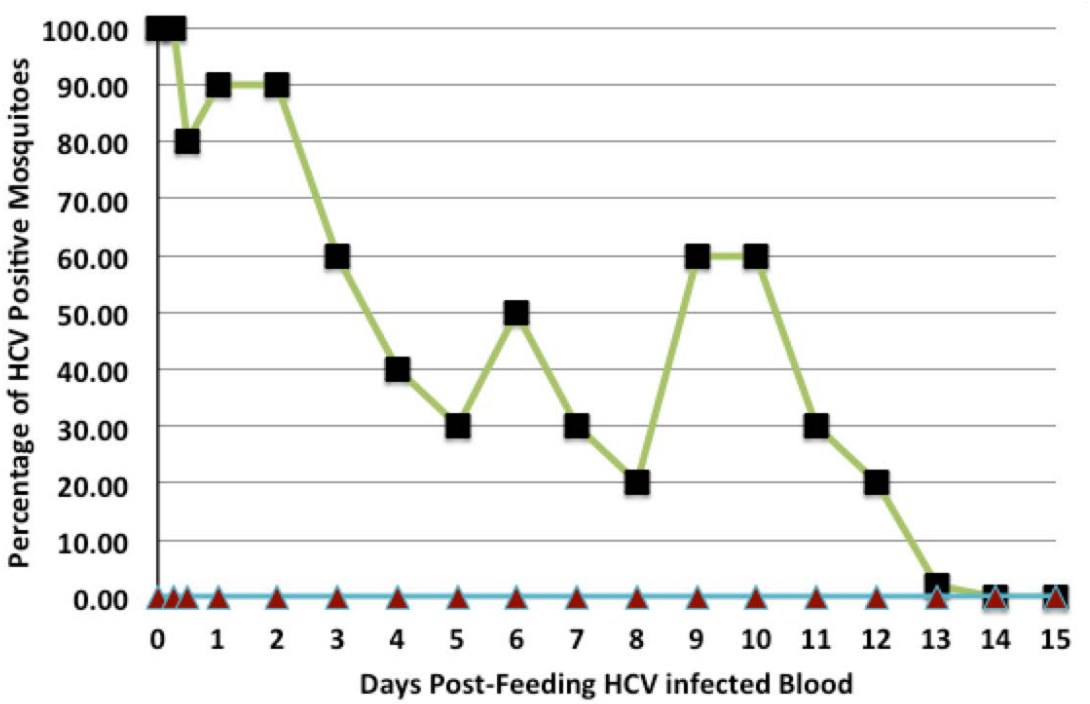

Figure (4): Clearance curve of viral genome from mosquitoes fed on infected blood. Ten mosquitoes (squares) were examined at each point for the presence or absence of HCV genomic RNA. Control (triangles) included mosquitoes fed on uninfected blood only.

Discussion

The current study involved an experimental investigation of feeding *Culex* mosquitoes on infected blood with HCV genotype four of a known viral concentration of (1.3 x10^5 IU/ml). Mosquitoes were then collected at different time points after the initial feeding and subjected to RT-PCR analysis. Results showed that all mosquitoes collected at time zero (directly after feeding) and 6 hours post-feeding were positive for HCV genome using RT-PCR analysis. Viral nucleic acid was detected in 8 out of 10 mosquitoes that were collected at 12 hours post-feeding. Nine from each of the ten of mosquitoes which were collected at the times 1 and 2 days after feeding were detected as having HCV nucleic acid, 2 out of 10 mosquitoes were still harboring the virus until day 13th after feeding with the infected blood. HCV nucleic acid was not found in the mosquitoes tested on day 14 and 15 after feeding.

Observations similar to our finding have been reported previously (10,13). However, there were some variations in the length of the period at which the virus can be recovered from mosquitoes after feeding on infected blood. For instance, (13) has reported that 100% (40/40) of *Culex* mosquitoes collected on day one after feeding had HCV and this ratio was reduced until day 25 after feeding to about 13% of the mosquitoes were positive for the virus. The variation in the longer persistence of HCV shown (25 days) in comparison to that reported in our study (13 days) may be attributed to many factors including but not limited to the number, the species of the *Culex* mosquitoes, viral genotype, temperature and the environmental factors of the insect breeding which was not mentioned in the above study.

Likewise, in another study (10), in which same genotype (genotype 4) was used, it showed that 100% of mosquitoes collected on day one post-feeding with infected blood harbor HCV virus genome. However, this percentage decreased with time and the virus disappeared on day 9 post-feeding with HCV infected blood. The disappearance of the virus is more quicker when compared to that observed in our study. This may be attributed to a possibly lower HCV titer in the blood used for feeding than that used in our study as the investigators did not specify the amount of virus used in feeding mosquitoes or due to different conditions of inset breeding and experimental protocols. It has also been proposed that persistence of the virus inside *Culex* mosquitoes is directly related to the presence of symbiotic bacteria as HCV fails to survive longer in mosquitoes that do not harbor such bacteria (10).

In this study we also investigated the infectivity through mouthparts of female mosquitoes, previously fed on HCV infected blood, after maintaining their feed via Parafilm on non-infected blood placed in Petri dishes. In those studies no HCV genomic RNA was detectable when using conventional RT-PCR analysis on samples collected from Petri dishes at days 2 through day 8 after feeding. Similar result was observed by (13) who have used a different method for evaluation of the infectivity through mouthparts. The procedure implemented can be described as female mosquitoes' proboscises washing technique and showed negative results.

It is worth mentioning that the lower detection limit of the conventional RT-PCR test used in both studies described herewas not less than 50 viral copies / ml of blood, that mean that: a fewer number is undetectable limit by this assay but it can induce infection. One viral particle perhaps is sufficient to induce infection through parenteral route (19).

Insects can transmit some pathogens incidentally, for instance, myxoma virus of rabbit which is not an arbovirus, can be transmitted mechanically by mosquito after its mouth parts become contaminated after feeding on an infected rabbit with skin lesions (20). Transmission of HCV from person to person via contaminated proboscises is plausible. Feeding of mosquitoes is a complex process (21) at which fluid exchange occurs between the insect and its host. The insect inserts their saliva inside the host prior to and during blood feeding. Such process may present a good chance to virus transmission. Furthermore, the skin of an individual may become contaminated with HCV when certain individual kill or destroy the mosquito that harbor the virus, thus the contaminated hands act as a source of viral transmission through skin cuts. This may explain the high percentage of asymptomatic HCV infections among population with no history of parenteral exposure to contaminated blood or other sources of the viral transmission.

It is worth mentioning that the role of insect transmission of pathogens not only involve inoculation of pathogens through mouth parts and insect saliva but also involve contamination of the insect bite sites by their containing the infectious agents. This is true as in the case of *Rickettsia prowazekii*, which is transmitted by feces of infected lice and and also in the case of *Trypanosoma cruzi* that is excreted in feces of the reduviid bug (22, 2). It is of interest to note that (23) have recorded that hepatitis B virus (HBV) can persist and vertically transmitted to the next generation of *Culex quinquefasciatus* when experimentally fed on infected blood. Also (24) reported that HBV can persist and be shed in the fecal droplets of the bed bugs *Cimex lectularius* for up to 35 days after experimental feeding on an infectious blood meal.

References

1. Choo Q. L; Kuo G; Weiner, A. J; Overby, L.R; Bradley, D. W. and Houghtan, M. (1989a). Isolation of cDNA clone derived from a blood-borne non-A, non-B viral hepatitis genome. Science. 244:395-62.
2. Alter, H. J; Purcell, R. H. and Shih, J. W. (1989) Detection of antibody to hepatitis C virus in prospectively followed transfusion recipients with acute and chronic non-A, non-B hepatitis Nat. Engl. J. Med. 321: 1494 500.
3. Greenwood, D; Slack, R; Peuther, J. B. and Barer, M. (2007). Medical Microbiology. 17th Edit. Elsevier Churchill Livingstone. Pp..
4. Sy, T. and Jamal, M. M. (2006). Review: Epidemiology of Hepatitis C Virus (HCV) Infection. Int.J.Med.Sci. 3(2): 41-46.65-69 .
5. Karaca, C; Cakalogu, Y; Demir, K; Ozdel, S; Kanymackoglu, S; Badur, S. and Okten, A. (2006). Risk factors for the transmission of hepatitis C virus infection in the Turkish population. Diges.Dis.Scin. 51(2): 3
6. Indolfi, G. and Resti, M. (2009). Review: Perinatal transmission of hepatitis C virus infection. J. Med. Virol. 81(5): 836-43.
7. Curry, M. P, and Chopra, S. (2005). Acute viral hepatitis . In : Mandell, G. L; Bennet, J. E. and Dolin, R. Editors. Principles And Practice of Infectious Diseases. Elsevier Churchill Livingstone. P. 1426-41.
8. Hayashi, J. and Furusyo, N. (2010). Review: Epidemiological and clinical study for hepatitis C virus infection. Semin. Liver. Dis. 30(3): 236-45.
9. Germi, R; Crance, J. M; Grain, D; Guimet, J; Thelu, M. A; Jouan, A; Zarski, J. P. and Drouet, E. (2001a). Mosquito cells bind and replicate hepatitis C virus. J. Med. Virol. 64(1): 6-12.
10. Hassan, M. I; Hammad, K. M; Amin, I; Mangoud, A. M; Etewa, S; Basheer, Z. M. and Sale-Arafa, W. A. (2002). Hepatitis C virus and *Culex pipiens* complex. J. Egyp. Soc. Parasitol. 32(3): 1003-14.
11. Pybus, O. M; Markov, P. V; Wu, A. and Tatem, A. J. (2007). Investigation the endemic transmission of the hepatitis C virus. Int. J. Parasitol. 37(8-9): 839-49.
12. Silverman, A. L; McCray, D. G; Gordon, S. C; Morgan, W. T. and Walker, E. D. (1996). Experimental evidence against replication or dissemination of hepatitis C virus in mosquitoes (Diptera : Culicidae) using detection by reverse transcriptase polymerase chain reaction. J. Med. Entomol. 33(3): 398-401.
13. Chang, T. T; Chang, T. Y; Cheng, C. C; Young, K. C; Roan, J. N; Lee, Y. C; Cheng, P. N. and Wu, H. L. (2001). Existence of hepatitis C in *Culex quinquefascintus* After ingestion of infected blood : Experimental approach to evaluating Transmission by mosquitoes . J. Clinicl. Microbiol. 39(9): 3353-55. -33.
14. Hassan, M. I; Mangoud, A. M; Etewa, S; Amin, I; Morsy, T. A; El-Hady, G; El-Besher, T. and Hammad, K. M. (2003). Experimenta demonstration of hepatitis C virus (HCV) in an Egyptian strain of *Culex pipiens* complex. J. Egyp. Soc. Parasitol. 32(2): 375-84.
15. Mohsen, Z. H. and Mehdi, N. S. (1989). Effect of insect growth inhibitor alsystin on Culex quinquefasciatus say. (Diptera : Culicidae). Insec. Sci. Appl. 10(1): 29
16. Al-Sharook, Z. M; Balank, K; Jiang, Y. and Rembold, H. (1991). Insect growth inhibitors from two tropical Meliaceae effect of crude extracts on mosquito larvae. J. Appl. Entomol. 111: 425-30.
17. Kauffman, E. B; Jones, S. A; Dupuis, A. P; Ngo, K. A; Bernard, K. A. and Kramer, L. D. (2003). Virus detection protocols for West Nile Virus in vertebrate and mosquito specimens. J. Clin. Microbiol. 41(8): 3661-67.

18. Zinser, M; Ramberg, F. and Willott, E. (2004). Culex quinquefasciatus (Diptera: Culicidae) as a potential West Nile Virus vector in Tucson, Arizona: Blood meal analysis indicates feeding on both humans and birds. J. Insect. sci. 20(4): 1-3.

19. Kamal, S. M. (2008). Acute hepatitis C: a systematic renview. Am. J. Gastroenterol. 103(5): 1283-97.

20. Strauss, J. H. and Strauss, E. G. (2002). Viruses And Human Disease. 1st Ed. Academic Press. P. 233.

21. Hubalek, Z. (2008). Review: Mosquito-borne viruses in Europe. Parasitol. Res. 103(1): 29-43.

22. Bowmann, D. D. and Lynn, R. C. (1995). Georg's parasitology For Veterinarians. 6th ed. W. B. Saunders company. Philadelphia. Pp.

23. Fouche, A.; Crewe, R. M. and Windsor, I. M. (1989). Vertical transmission of hepatitis B virus in *Culex quinquefasciatus*. Med. Vet. Entomol. 3(4): 385-89.

24. Blow, J. A; Turell, M. J; Silverman, A. L. and Walker, E. D. (2001). Stercorarial shedding and transtadial transmission of hepatitis B virus by common bed bugs (Hemiptera: Cimicidae). J. Med. Entomol. 38(5): 694-700.