EXPERIMENTAL EVALUATION OF THE ROLE OF SYMBIOTIC AND APOSYMBIOTIC, *CULEX PIPIENS* MOSQUITOES IN THE TRANSMISSION OF HEPATITIS C VIRUS (HCV)

By

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Abstract

The present study evaluated the potential role of *Culex pipiens* mosquitoes in the transmission of HCV, post being fed on an infected blood with a viral load of 1.3×10^6 IU/ml.in symbiotic mosquitoes and 1.2×10^6 IU/ml.in aposymbiotic one. Mouth parts, mid-gut and salivary glands in both symbiotic and aposymbiotic mosquitoes at different time intervals were tested for evaluation of viral load using real-time PCR for up to 60min and up to 5, 13 day in case of mouth parts, mid-gut and salivary glands respectively. It was observed that the viral load was decreased by increasing the time post feeding in mid-gut and mouth parts in both symbiotic and aposymbiotic mosquitoes so, there is a negative relationship between the HCV load and feeding time. On the other hand, HCV was not detected in salivary glands in both symbiotic and aposymbiotic mosquitoes during the period of detection. The results showed that the mechanical transmission through mouth parts in both symbiotic and aposymbiotic mosquitoes are plausible, while biological transmission through symbiotic and aposymbiotic mosquitoes did not occur.

Key words: *Culex pipiens,* Symbiotic, Aposymbiotic, HCV, Transmission.

Introduction

The mosquitoes are vectors of many vertebrate blood parasites. In Egypt *Culex pipens* has a wide distribution and is the main vector of Rift Valley Fever (RVF) virus (Megan *et al*, 1980; Darwish and Hoogastraal, 1981; El Bahnasawy *et al,* 2013a), *Wuchereria bancrofti* (Khalil *et al*, 1930; Gad *et al,* 1996; Abdel-Hamid *et al*, 2013) and Western Nile Virus (Pelah *et al*, 2002; El Bahnasawy *et al*, 2013b). Hassan *et al.* (2003); Pybus *et al.* (2007); Tarish *et al.* (2014) and El Kholy *et al.* (2017) studied the possibility of the HCV experimental transmission by different mosquitoes.

Hepatitis C Virus infection is one of the major public health problems in both developed and developing countries since discovering at 1989 (Choo *et al,* 1989; Alter *et al,* 1989). It was 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 (Sy and Jamal, 2006). The infection acquired mainly via parenteral route (Karaca *et al,* 2006), and also perinatally (Indolfi and Resti, 2009), but there is (30-40%) of infected cases were without identifiable route (Hayashi and Furusyo, 2010).

Many investigators suggested that the mechanical transmission of HCV by mosquitoes was plausible (Germi *et al,* 2001; Hassan *et al,* 2003; Pybus *et al,* 2007). This was in accordance to Kamal (2008) who reported that one viral particle was perhaps sufficient to induce infection via parenteral route. El Kholy *et al.* (2017) reported that the mosquito *Cx. pipiens* may be a potential vector of HCV. However, the biological and mechanical of HCV transmission by mosquitoes showed negative results. Low HCV RNA titers in patient sera and different species tropisms for HCV/RNA replication are probably the reasons why the mechanical and biological HCV transmission did not occur in mosquitoes (Chang *et al*, 2001).

Bacteria exist naturally in the mid-gut of wild and laboratory-reared mosquitoes (Chao and Wistreich, 1960; 1995; Demaio *et al*, 1996; Pumpuni *et al*, 1996) and sand flies (Schlein *et al*, 1985; Hassan *et al*, 1998). Bacteria occurring in gut of haematophagous insects may have an important role in epidemiology of human infectious diseases. Such bacteria may interfere with the development of medically important pathogens. The high midgut counts of Gram-negative bacteria significantly reduced oocysts in infected *Anopheles* (Seitz *et al*, 1987; Beier *et al*, 1994; Pumpuni *et al*, 1996).

The present study aimed to evaluate the role of symbiotic and aposymbiotic *Culex pipiens* mosquitoes in the transmission of Hepatitis C virus (HCV) even biologically or mechanically.

Materials and Methods

Mosquitoes rearing technique*:* Mosquito used was *Culex pipiens* L., obtained from the Medical Entomology Institute Dokki, Giza. *Culex* was reared for several generations, in the Insectary of Medical Entomology at the Department of Zoology, Faculty of Science, under controlled laboratory conditions at temperature of 27±2°C, relative humidity 70±10% and 12-12 light-dark regime.

Elimination of bacteria: In order to eliminate mi d-gut bacteria, *C. pipiens* females were fed on sponge piece soaked in a feeding medium, which composed of 10gm. sucrose and 0.014g of a wide spectrum antibiotic (Tarivid) dissolved in 100ml. distilled water. The mixture was kept in a refrigerator at 4°C. A sterile sponge piece was saturated with the mixture and offered daily to *C. pipiens* females. This process was continued for a period of 3 days after adult emergence. This group of insects was used for aposymbiotic test (Fouda *et al,* 2013).

Experimental infection of with HCV: All experiments were carried out at Genetic Engineering Center, Faculty of Science, Al-Azhar University under controlled laboratory conditions.

Feeder membrane preparation: The feeder membrane (Fig. 1) consisted of two tubes, first one for positive blood meal surrounded with the second one for the current water which connected with water bath at 37°C to keep the blood temperature. The 1st tube with two ends, the bottom, covered with chicken membrane and the upper covered with a piece of cotton after a blood meal entered. The 2nd tube with two ends also, for income and outcome the current water.

Fig.1: Feeder membrane equipment

Blood preparation: Two blood bags were brought from Blood Bank at VACCERA, Dokki, Giza-Egypt, post signing approval of blood bank QC Department. Blood bags were tested for positive reactivity to HCV virus titer using PCR to estimate the viral load. Preparing and dissecting samples: Mosquitoes were kept in feeding cages and they were provided daily with 10% sucrose solution for a period of one day after emergence. The sugar solution was removed 24 h before the blood meal to increase blood-feeding. After that; the females were allowed to take a positive blood meal via the feeder membrane, then the specimens were dissected and prepared (Fouda *et al,* 2013).

Viral load in different parts of dissecting mosquitoes: *Culex pipiens* were collected for viral load detection in mouth parts, at once, 30min, and 60min, mid-gut at once, after 12 hr, 1st, 2nd, 3rd, 4th & 5th days and in salivary glands at 5, 6, 11, 12, & 13 days after feeding on HCV infected blood respectively. Killed mosquito mouth parts, mid gut and

salivary glands were placed in a little amount of normal saline solution under freezing conditions to be examined using real-time PCR.

Results

Evaluation of infectivity titer in mouth parts of test mosquitoes: There was a decrease in HCV titer percentage induced by increasing post feeding time in both symbiotic and aposymbiotic *C. pipiens* mouth parts. There was a negative correlation between HCV titer and time post feeding (Tab. 1, Fig. 2). There was a depletion of viral load in infected blood fed aposymbiotic *C. pipiens* than in symbiotic ones at zero time. There was an elevate viral load in aposym-

biotic *C. pipiens* 30min. post feeding and opposite was clear one hr post feeding in symbiotic ones. The mechanical transmission through mouthparts of symbiotic and aposymbiotic *C. pipiens* might be plausible.

There was a decrease in HCV titer by increasing time, with a negative correlation between HCV titer and time (Tab, 2; Fig. 3) but, an increase in viral load in mid gut of aposymbiotic *C. pipiens* than symbiotic one related to time/day. Also, no HCV titer at 5, 6, 11, 12 & 13 days post feeding, without correlation between time and titer. So, no biological transmission occurred via symbiotic and aposymbiotic *C. pipiens*.

Table 1: HCV titer at mouth parts in symbiotic and aposymbiotic mosquitoes relative to time post feeding.

Post-feeding (min)	Tested mosquitoes	Symbiotic HCV titer IU/ML% /10 females	Symbiotic HCV titer/ female %	Aposymbiotic HCV titer IU/ML% /10 females	Aposymbiotic HCV titer / female %
0	10	116122	11612.2	100129	10012.9
30	10	36046	3604.6	46548	4654.8
60	10	1381	138.1	_	_

Tab le 2: HCV titer in the mid-gut of symbiotic and aposymbiotic mosquitoes relative to time post feeding.

Post-feeding (days)	Tested mosquitoes	Symbiotic HCV titer IU/ML%./10 females	Symbiotic HCV titer/female %	Aposymbiotic HCV titer IU/ML%/ 10 females	Aposymbiotic HCV titer/female %
0	10	244336	24433.6	299576	29957.6
0.5	10	141051	14105.1	210993	21099.3
1	10	129093	12909.3	160737	16073.7
2	10	98302	9830.2	148019	14801.9
3	10	12348	1234.8	106889	10688.9
4	10	1641	164.1	85790	8579
5	10	1073	107.3	74789	7478.9

Table 3: HCV titer in the salivary glands of symbiotic and aposymbiotic mosquitoes relative to time post feeding.

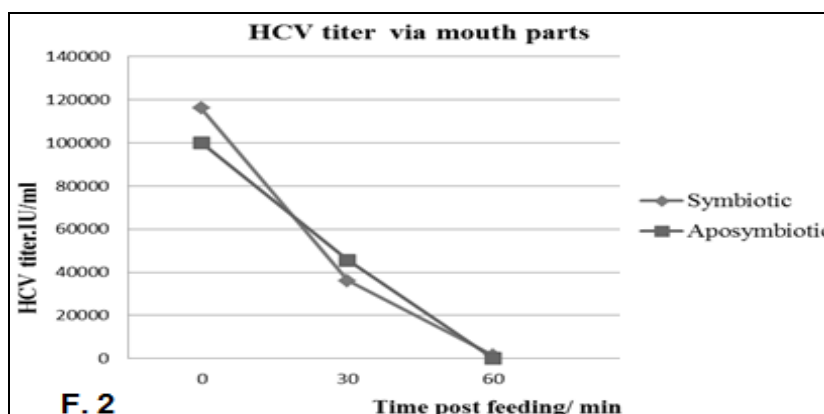| Post-feeding (days)	Tested *C. pipiens*	Symbiotic HCV titer IU/ML%/ 10 females	Symbiotic HCV titer % / female	Aposymbiotic HCV titer IU/ML% per 10 females	Aposymbiotic HCV titer %/ female
5	10	_	_	_	_
6	10	-	-	-	-
11	10	-	-	-	-
12	10	-	-	-	-
13	10	-	-	-	-

Fig. 2: HCV genome of mouth parts from symbiotic and aposymbiotic *C. pipiens* fed on infected blood at different intervals post feeding.
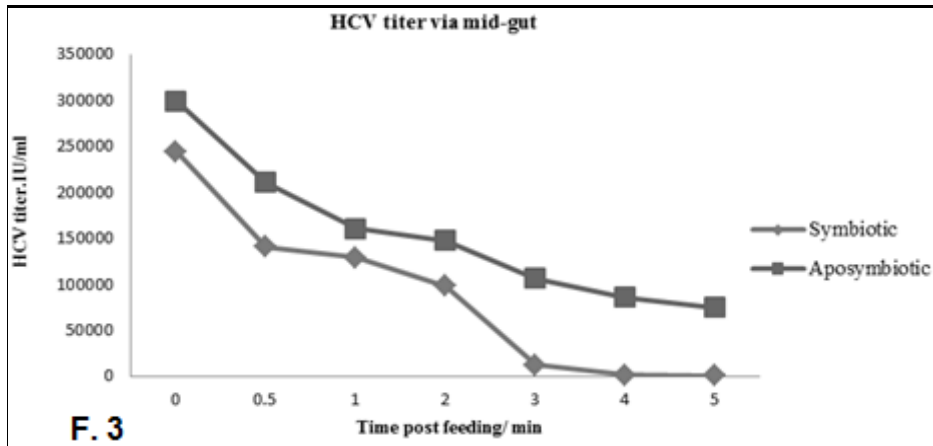
Fig. 3: HCV genome in mid-gut of symbiotic and aposymbiotic *C. pipiens* fed on infected blood at different intervals post feeding.

Discussion

The current study investigated feeding *C. pipiens* on infected blood via feeder membrane with HCV genotype IV of a known viral concentration of $(1.3 \times 10^6 IU/ml)$ in blood meal of symbiotic mosquitoes and with viral concentration of $(1.2 \times 10^6 IU/ml)$ in blood meal of aposymbiotic one. The results showed that all mosquitos' moth parts examined at time zero, 30 min and 60 min post-feeding were positive for HCV genome using real time-PCR analysis. These results were in harmony with Hassan *et al.* (2003) and Tarish *et al.* (2014) despite of presence of some variations in the period length at which the virus was recovered from mosquitoes after feeding on infected blood. Hassan *et al.* (2003) reported that HCV-RNA was detected in heads of symbiotic females at 3hr post feeding and in aposymbiotic ones the virus was detected afterwards in head and guts. The procedure implemented can be described as the variation in the longer persistence of HCV shown (3 h) in comparison to that reported in the present study (1 h) may be attributed to many factors including the viral genotype, temperature and the environmental factors affected insect breeding but not limited to the virus titer.

Also, Tarish *et al.* (2014) investigated the infectivity through mouthparts of female *Culex* spp. 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 2 to 8 days port-feeding.

For the mechanical transmission to occur from an infective insect vector to a human host, infectious viral units on the mouth parts must come in contact with the healthy susceptible person through direct percutaneous exposure at the moment of insect probes the human skin. Such an event might occur when mosquito is disturbed while feeding on a person, when it flies to another person for feeding i.e. interrupted feeding (Sliver *et al*, 1996). Transfer equine infectious anemia virus from one horse to another by biting flies is a well-established mode of such mechanical transmission (Foil and Issel, 1991). Whether HCV can be transferred from an infected person to healthy one by this route of infection is still unknown.

However the present results showed a negative correlation between the HCV titer and time in symbiotic and aposymbiotic mosquitoes. Although the titer decreased when the time increased, it may contribute and raise probability of mechanical transmission of HCV. Thus, explained percent (30-40%) of infected cases are without identifiable route (Hayashi and Furusyo, 2010). This agreed with Kamal (2008) who reported that one viral particle perhaps sufficient to induce infection through parenteral route.

Insects can transmit some pathogens incidentally, for instance, myxoma virus of rab-

bit which is not an arbovirus, can be transmitted mechanically by mosquito after its mouth parts become contaminated after being fed on the infected rabbit with skin lesions (Strauss and Strauss, 2002). Transmission of HCV from person to person via contaminated proboscises was plausible (Tarish *et al,* 2014). Feeding of mosquitoes is a complex process at which fluid exchange occurs between the insect and its host. The insect inserts her 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 without history of parenteral exposure to contaminated blood and/or other sources of the viral transmission (Hubalek, 2008).

In the present study, all the experiments showed a decrease in the virus titer by the time, with variation in the titer levels in aposymbiotic compared to symbiotic ones due to absence of mid-gut bacteria. The results agreed with Seitz *et al*. (1987); Beier *et al*. (1994) and Pumpuni *et al*. (1996) who reported that bacteria occurring in the gut of haematophagous insects may have an important role in epidemiology of human infectious diseases. Such bacteria may interfere with the development of medically important pathogens.

The prevalence of biologically transmitted pathogens in vectors can be low even though the pathogen might live in the vector for many days (Anderson and May, 1991). The low transmission coefficients that Pybus *et al*. (2007) reported the rapid decay of HCV-virus within a mechanical vector, suggested that prevalence of HCV infection amongst vectors was exceedingly low.

The present results agreed with Chang *et al*. (2001) who showed that the biological

HCV transmission by *C. quinquefasciatus* did not occur. Low HCV/RNA titers in patient sera and different species tropisms for HCV RNA replication are probably the reasons why biological HCV transmission did not occur in *C. quinquefasciatus*.

In the present study, all results showed negative HCV/RNA. These results agreed with Chang *et al*. (2001) who infected *C. quinquefasciatus* by intrathoracic inoculation using pulled disposable pipette needles to avoid possible mid-gut HCV barriers and even though the biological HCV transmission by *C. quinquefasciatus* was negative. The results showed that *C. pipiens* failed to be HCV biological transmitter.

Hassan *et al,* (2003) *C. pipiens* trapped from of HCV patients' homes or among the same organs of symbiotic (normal gut bacteria) and aposymbiotic (without gut bacteria) mosquitoes fed HCV positive blood by an artificial membrane feeder. They found by PCR, HCV/RNA only in heads of symbiotic mosquitoes collected from homes of HCV positive patients at 3hr and 6hr post-feeding. Virus was detected at 3rd & 8th days post-fed in guts of the same group. They raised the possibility of the mechanical and/or biological transmission of HCV by *C. pipiens*, and pave the way to study the effect of gut bacteria in a trial to identify an anti-HCV agent. Also, El Kholy *et al*. (2017) reported that HCV positive strands were detected in salivary glands of mosquito adults at a week post-feeding

For the biological transmission to occur from an infective insect vector to man, the virus must be replicated such as mosquito- and tick-borne flaviviruses are zoonotic transmitted through haematophagous insects during blood meal feeding. Viruses obtained from vertebrate host initially replicated in the mid-gut within 5-7 minutes of exposure and then virus spreads to other tissues via haemolymph and transmitted with saliva during blood meal (Blair *et al*, 2000). The period from the initial infection in the mid-gut to when the vector transmits the virus is

termed the extrinsic incubation period (EIP), and this time period varies from7 to 14 days (Ramirez and Dimopoulos 2008). However, viruses were detected in the salivary glands of DENV-infected *Aedes aegypti* as early as 24hrs (Luplertlop *et al*, 2011), Urban Chikungunya (Humphrey *et al*, 2017), Yellow Fever virus (Klitting *et al*, 2018), as well as Zika virus (Tham *et al*, 2018). Consequently, the EIP depended on the virus, the mosquito and certain environmental factors (Ramirez and Dimopoulos, 2010).

Generally speaking, the major role in the transmission of HBV and/or HCV infection was played by the virus concentration on the infecting materials, high in blood and blood products, much lower in ascites and in cerebrospinal fluid and at non-infectious concentrations in feces, urine, sweat, vomit and tears (Coppola *et al*, 2016). Undoubtedly, the HBV and HCV are blood-borne infections, with significant transmission occurring through unsafe injections and medical procedures, and less commonly by sexual contact (Demsiss *et al*, 2018).

The health-care workers worldwide are, thus, at greater risk of acquiring these two blood-borne infections from percutaneous (needle-stick/sharp instrument) injuries or other types of occupational exposures, and the incidence of this infection among them has been estimated to be four times the level in the general population (Ciorlia and Zanetta, 2005). Needle-stick accidents with percutaneous inoculation is a well-documented HBV and HCV transmission, with seroconversion rate of 10-30% following needle stick exposure to HBV infected blood and about 2.0% for HCV (Tarantola *et al*, 2005). Even in malaria which used the female *Anopheles*-vector for cyclopropagative biological transmission, was proved to be occupational needle-stick injury (Abdel-Motagaly *et al*, 2017). Overall the number of the health-care workers annually exposed to the different sharp injuries contaminated with HBV and HCV was estimated to reach 2.1 million and 926,000, respectively (Prüss-

Ustün *et al*, 2005). The protection of health care workers via immunization (for HBV), use of protective equipment and post-exposure management was therefore very critical to prevent occupational risk of exposure to the transmission of these blood-borne viruses (Deuffic-Burban *et al*, 2011).

Egypt was experiencing an HCC epidemic largely due to widespread HCV infection (Eldin *et al,* 2014), with the world's highest infection prevalence (~10%) in 2008, and a 2015 HCV seroprevalence of 6.3% (Egypt Health Issues Survey, 2015). HCV transmission was still ongoing in Egypt, with new cases estimated at 2.4/1000 person-years (El-Akel *et al*, 2017). Nowadays, the use of sofosbuvir-related therapies producing improved treatment success had permitted an updated, nationwide, HCV treatment programme with the expanded coverage (Ma *et al*, 2018).

Conclusion

The outcome results showed that the mechanical transmission via mouth-parts of the symbiotic and/or aposymbiotic female *Culex pipiens* might be plausible. Biological transmission via salivary glands of *C. pipiens* symbiotic and aposymbiotic did not occur.

Nevertheless, the accidental needle-stick injuries must be in mind of all the healthcare workers.

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