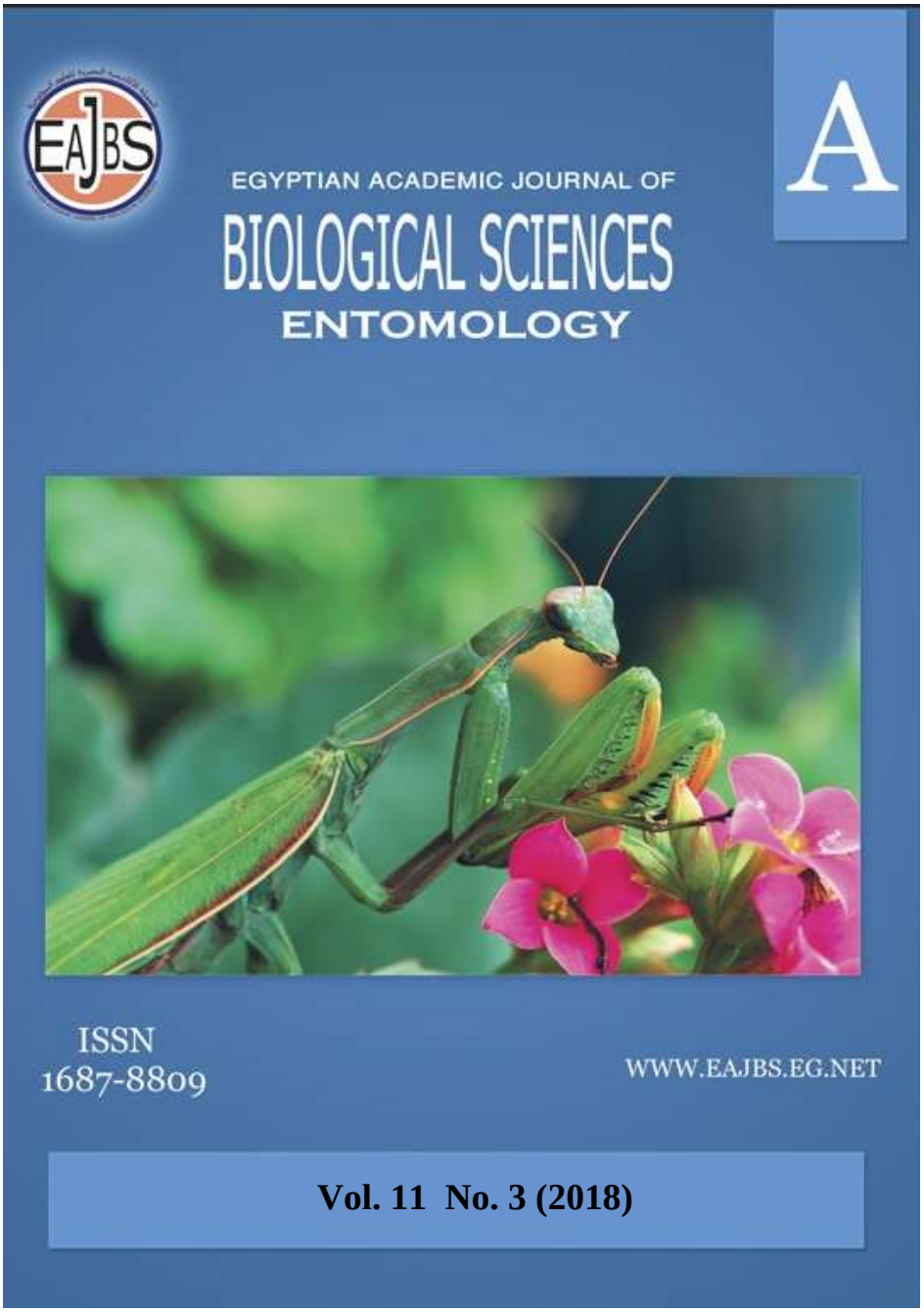

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Evaluation of the Role of Irradiated, *Culex pipiens* , Mosquito (Diptera; Culicidae) in the Transmission of Hepatitis C Virus (HCV)

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ABSTRACT

In the present study, gamma radiation effects on the reproductive potential of the mosquito, *Culex pipiens* and its role in the transmission of the Hepatitis C Virus (HCV) were investigated. The susceptibility of female, *Culex pipiens* mosquitoes to gamma irradiation was carried out by exposed full grown pupae to doses 0, 20, 40, 60 Gy. The lethal doses were calculated, as the doses of gamma radiation increased, a progressive increase in the non-hatched eggs percentage. The viral load at mouth parts, in mid-gut and salivary gland, was detected in the irradiated females with LD_{75} (60 Gy) and non-irradiated using RT-PCR relatively at time 60 min., 5 and 13 days and tested in the mentioned position. Viral load in irradiated *Culex pipiens* that fed on an infected blood with a viral load 1.2 x 10^6IU/ml **%**was decreased by time from 6.0782 x 10^4 IU/ML% at zero time into 2.399 x 10^3 IU/ML% after 60 min. post-infection at the mouth parts. Also, the viral load decreased by time in the mid-gut from 2.63575 x 10^5 IU/ML% at zero time into 3.969 x 10^3 IU/ML% after 5 days post-infection, while HCV was not detected in the salivary glands. The current results indicated that the mechanical transmission through mouth parts in irradiated and non-irradiated *Culex pipiens* mosquitoes is plausible while the biological transmission did not occur.

INTRODUCTION

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 is estimated that HCV infects 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 through parenteral route (Karaca *et al.*,2006), and also perinatally (Indolfi and Resti 2009), but (30-40%) of infected cases are without identifiable route (Hayashi and Furusyo 2010). One of the suspected routes of HCV transmission refers to activity by blood-sucking insects, such as female mosquitoes, which take blood meals from human hosts. In this context, several flaviviruses, such as yellow fever, Japanese

encephalitis and dengue viruses, have been found to be transmitted by mosquitoes (Lundström, 1999; Bakonyi *et al*., 2005).

Many investigators have suggested that the mechanical transmission of HCV by mosquitoes is plausible (Germi *et al.,* 2001; Hassan *et al.,* 2003; Pybus *et al.*,2007). Others reported that 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 mechanical and biological HCV transmission does not occur in mosquitoes so it seems mosquitoes is not an HCV risk factor. (Chang *et al*., 2001)

Radiation effects on the molecular chemical species of the living organism body will provide information that is essential to the knowledge of the radiation-induced molecular alteration that will initiate the biological chain of damage and its final sequel. (El-Naggar, 2009). Protein, lipid and carbohydrate molecules are the essential nutritive components in the biological system in insects. Therefore, any disturbance in this component leads to the disturbance in the biological system and adult performance (Gabarty and Mahmoud, 2015).

The aim of this work is to evaluate the susceptibility of female *Culex pipiens* mosquitoes to gamma rays, and the role of irradiated and non-irradiated mosquitoes in the transmission of hepatitis C virus (HCV).In addition, is there any conflict between the usage of gamma rays in the control of *Culex pipiens* and their role in the transmission of (HCV) in comparison to the non- irradiated insects.

MATERIALS AND METHODS

Insect Rearing Technique:

Culex pipiens was obtained from the Medical Entomology Institute. It was reared for several generations, in the Insectary of Medical Entomology at the Department of Zoology and Entomology, Faculty of Science, Al-Azhar University, under controlled laboratory conditions at the temperature of 27±2°C, relative humidity 70±10% and 12-12 light-dark regime.

Irradiation Process:

Females' full grown pupae were collected daily by pipette and kept in glass dechlorinated tap water for irradiation. The irradiation process was performed using Gamma cell-40 (cesium-137 irradiation unit), at National Center for Radiation Research and Technology, Cairo (NCRRT). The dose rate was 0.688 Rad/sec at the time of the present investigation.

The Susceptibility of Females' *C. pipiens* Resulted From Irradiated Pupae to Gamma Radiation:

Females' pupae were irradiated with 4 doses 0, 20, 40, and 60 Gy, Fecundity (No. of eggs laid), Fertility (No. of eggs hatched) were detected in the developed females. The percentages response of non- hatching eggs was corrected using Abbotts' Formula (Abbott, 1925). The (LD_{25}), (LD_{50}), (LD_{75}) and (LD_{90}) were calculated according to the method of (Finney, 1971).

Two crossing combinations was set up for each dose as follows: 4 replicates of resulted in irradiated ♀♀X non-irradiated ♂♂ and 4 replicates of non-irradiated ♀♀X non-irradiated ♂♂ were used as the control.

Egg – Hatchability:

The eggs were sorted into two categories: hatched and non-hatched eggs according to the method used by Hassan *et al*., (1996).The egg-hatchability was calculated by using the following equation:

Egg-hatchability % = A / B × 100

A = Total No. of hatched eggs. B = Total No. of eggs laid.

Experimental Infection of *C. pipiens* with Hepatitis C Virus:

All experimental infection was carried out at Genetic Engineering Center, Faculty of Science, Al Azhar University under controlled laboratory conditions.

Feeder Membrane Preparation:

Feeder membrane (Fig. 1) consists of two tubes, the first one for positive blood meal surrounded with the second one for the current water which connected with the water bath at 37 ºC to keep the blood temperature. The 1st tube with two ends, the bottom, covered with chick 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 of the current water.

Fig. (1): feeder membrane equipment

Blood Bag Preparation:

HCV contaminated blood bags were supplied from the blood bank, donation unit VACSERA, Dokky, Giza-Egypt. Blood bag was examined by RT-PCR technology.

Preparing Samples:

Mosquitoes (control and irradiated females) were kept in feeding cages and they were provided daily with sponge pieces soaked in 10% sucrose solution for a period of 3-4 days after emergence. Mosquitoes were starved 24 h before the blood meal to increase blood-feeding through the feeder membrane. *C. pipiens* was fed on an infected blood with a viral load of 1.3 x 10^6IU/ml %.in control mosquitoes and of 1.2 x 10^6IU/ml %.in irradiated one.

Dissection of Infected Mosquitoes:

Mosquitoes collected by electric aspirator were kept in the feeding cage. The Specimens were dissected and prepared according to **(Fouda *et al.*, 2013).**

Infection Rate :

Ten females of *C. pipiens* mosquitoes for every maintenance time were dissected post feeding on blood meal and subjected for real-time PCR for detection of HCV. Infection rates were calculated as the following:

$$\text{Infection rate} = \frac{\text{No.of virus titer post feeding}}{\text{No.of virus titer before feeding}} \text{x } 100$$

Statistical Analysis:

All data obtained for (biological studies) were statistically analyzed and the variance ratios were calculated by the method of one way ANOVA using (SPSS/PC) computer program calculated at 5% level.

RESULTS

The Susceptibility of Females' *C. pipiens* Resulted from Irradiated Pupae to Gamma Radiation:

The effects of gamma radiation on fecundity of *C. pipiens* females resulted from pupae irradiated with 0, 20, 40 and 60 Gy and crossed with non-irradiated males are given in table (1). The number of eggs/10♀ decreased from 2444±22.65 at 0Gy to 311±15.20 at 60Gy. Gamma radiation affects the non-hatching egg percentage as compared with the control group. At the highest doses (60Gy) the non- hatching egg percent recorded 91.775%, while at the lowest doses (20Gy) recorded 36.782% table (1). From the aforementioned results, The LD_{25}, LD_{50}, LD_{75} and LD_{90} recorded 20, 35, 60 and 95 Gy, respectively, based on the susceptibility of non-hatching eggs percentage resulted from irradiated female crossed with normal male (Fig. 2 & Tab 2).

Table (1): Susceptibility of females' *C. pipiens* resulted from irradiated pupae to gamma radiation

Dose (Gy)	Total eggs count/10♀ (Mean ±SD)	Hatching eggs (Mean ±SD)	Non-hatching eggs (Mean ±SD)	Response % of non- hatching eggs	Corrected response%
0	2444±22.65	2177±37.22	267±10.30	10.993	0
20	348.15±18.21***	220.15±21.18***	128±6.33***	36.782	28.97
40	812.18±33.31***	369.18±28.17***	443±25.21**	54.557	48.94
60	311±15.20***	19±11.11***	292±15.21ns	91.775	90.75

Gy= Gray, SD = Standard deviation.

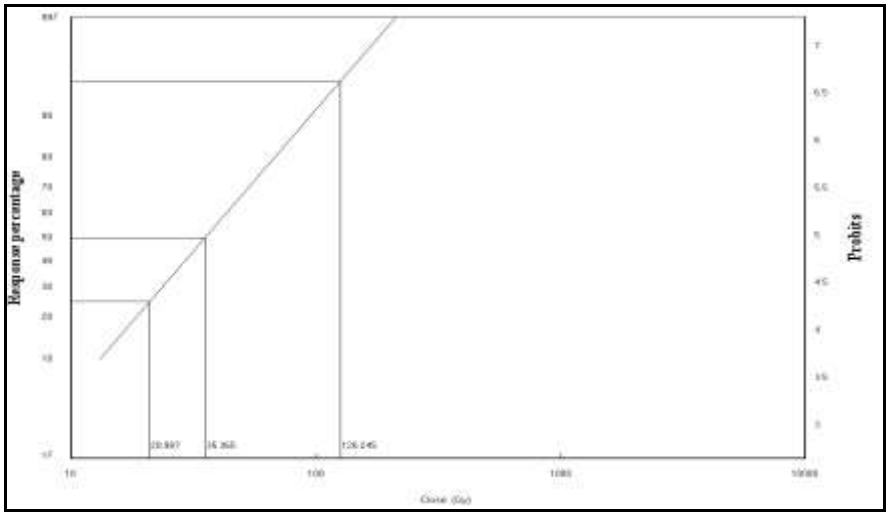

Fig. (2): Doses--- Probit lines of the non-hatching eggs response percent resulted from irradiated females of *C. pipiens*

Table (2): Lethal doses of gamma radiation affecting the non-hatching eggs of *C. pipiens* irradiated females crossed with non-irradiated males

(LD_{25}) in(Gy).	(LD_{50}) in (Gy).	(LD_{75}) in (Gy).	(LD_{90})in (Gy).	Slope ± S.E.
20	35	60	95	2.976±0.2297

S.E= Standard error

Evaluation of Infectivity Titer at Mouth Parts of Irradiated and Non-Irradiated *Culex pipiens,* Mosquitoes:

The data demonstrated that, there was a decrease in the percentage of the HCV titer which induced by increasing the time post-feeding at both control and irradiated mosquitos' mouth parts. A negative correlation between the HCV titer and post feeding time was observed in the table (3). Data showed that there was a decrease of viral load in infected blood fed irradiated mosquitoes than that in control mosquitoes at zero time and after 30 min. post feeding, while there was an increase in viral load in irradiated mosquitoes 60 minutes post feeding. From the fore mention results, mechanical transmission through mouthparts of control and irradiated mosquitoes may be plausible and this needs further work.

Table (3): HCV titer at mouth parts in irradiated and non-irradiated mosquitoes relative to time post feeding

Time after feeding (min)	No. of tested mosquitoes	HCV over load $1.3x10^6$ IU/ml	HCV over load $1.2x10^6$ IU/ml
		Non-irradiated HCV titer IU/ML%	Irradiated HCV titer IU/ML%
0	10	$1.16122 x10^5$	$6.0782 x10^4$
30	10	$3.6046 x10^4$	$3.812 x10^3$
60	10	$1.381 x10^3$	$2.399 x10^3$

Evaluation of Infectivity Titer in The Mid-Gut of Irradiated and Non-Irradiated *Culex pipiens,* Mosquitoes:

By evaluating the infectivity titer in mid-gut of irradiated *Culex pipiens*, mosquitoes, there was a decrease in the percentage of the HCV titer which induced by increasing the time post-feeding in both control and irradiated mosquitos' mid-gut. A negative correlation between the HCV titer and post feeding time was observed in the table (4). The infection rate was a variable decreased among irradiated mosquitoes vs. control mosquitoes by increasing the time post feeding. From aforementioned results it is obvious that there was a negative correlation between the infection rate and the time where it was 21.96 , 19.60 , 19.15 , 18.38 , 3.58 , 1.14 and 0.33% in irradiated mosquitoes, vs 18.79 , 10.85 , 9.93 , 7.56 , 0.94 , 0.12 and 0.08 % in control at times, 0 , 0.5 , 1, 2, 3, 4 and 5 days, respectively, (Tab 4).

Table (4): HCV titer and infection rate at mid-gut in irradiated and non-irradiated *Culex pipiens* mosquitoes relative to time post feeding

Time post feeding (days)	Number of tested mosquitoes	HCV over load $1.3x10^6$ IU/ml		HCV over load $1.2x10^6$ IU/ml	
		Non-irradiated HCV titer IU/ML%	I.R(%)	Irradiated HCV titer IU/ML%	I.R(%)
0	**10**	**$2.44336x10^5$**	18.79	**$2.63575 x10^5$**	**21.96**
0.5	**10**	**$1.41051 x10^5$**	10.85	**$2.35230 x10^5$**	**19.60**
1	**10**	**$1.29093 x10^5$**	9.93	**$2.29877 x10^5$**	**19.15**
2	**10**	**$9.8302 x10^4$**	7.56	**$2.20595 x10^4$**	**18.38**
3	**10**	**$1.2348 x10^4$**	0.94	**$4.3006 x10^4$**	**3.58**
4	**10**	**$1.641 x10^3$**	0.12	**$1.3776 x10^4$**	**1.14**
5	**10**	**$1.073 x10^3$**	0.08	**$3.969 x10^3$**	**0.33**

Evaluation of Infectivity Titer in The Salivary Gland of Irradiated and Non-Irradiated *Culex pipiens*, Mosquitoes:

By the final estimation of infectivity HCV titer in the salivary gland of irradiated and non-irradiated *Culex pipiens*, mosquitoes RT-PCR analyses, there was no HCV titer at 5, 6, 11, 12and 13 days post blood feeding, in turn there was no correlation between the time and HCV titer table (5). So, the recorded data revealed that the biological transmission via irradiated and non-irradiated mosquitoes did not occur.

Table (5): Evaluation the infectivity with HCV through the salivary glands in irradiated and non-irradiated *Culex pipiens* mosquitoes relative to time post feeding

Time post feeding (days)	Number of tested mosquitoes	HCV over load 1.3x106 IU/Ml%	HCV over load 1.2x106 IU/Ml%
		Non-Irradiated HCV titer IU/ML%	Irradiated HCV titer IU/ML%
5	**10**	--	--
6	**10**	--	--
11	**10**	--	--
12	**10**	--	--
13	**10**	--	--

DISCUSSION

Currently, exposure to ionizing radiation is the method of choice for rendering insects reproductively sterile for area-wide integrated pest management (AW-IPM) programs that integrate the sterile insect technique (SIT). So, we need in this study to discuss according to our result is there any conflict between the usage of gamma rays in the control of *Culex pipiens* and their role in the transmission of (HCV) in comparison to the non- irradiated insects.

Response % (non-hatching eggs percentage) laid by the irradiated female (emerged from irradiated pupae) mated with the non-irradiated male of the *Culex pipiens*, was found to be dose-dependent i.e. increased as the dose of the gamma irradiation used increased. The obtained data indicated that the highest non-hatching percent was caused by the highest dose (60 Gy), Meanwhile, the lowest dose (20Gy) caused lowest non-hatching percentage. In agreement with the present results, **Draz**

***et al.*, (2008)** irradiated 7-days-old male pupae of *Bactrocera zonata* (Saunders) with doses of 10, 30 and 50 Gy of gamma radiation. The percent of non-hatched egg ranged from 30.6 to 47.2 % for females mated with irradiated males with 10 Gy, Such percent were ranged from 94.34 to 99.96 % and 96.57 to 100.0 % for females mated with irradiated males with 30 and 50 Gy, respectively. Also **Resilva *et al.*, (2007)** reported that irradiation of *Bactrocera philippinensis* pupae with doses lower than 67 Gy did not prevent egg hatching. **Gabarty, (2011)** studied the effect of gamma radiation on the greasy cutworm *Agrotis ipsilon* and observed that the percentage of non- hatching eggs resulted from irradiated full grown male pupae was found to be dose-dependent i.e. increased as the dose of the gamma irradiation used increased. The highest percent of non-hatching eggs was caused by the highest dose (240 Gy), Meanwhile, the lowest dose (40Gy) caused the lowest percentage.

According to, the dose-response curves between dose and non- hatching eggs percentage we can indicate the accurate dose that prevents the hatchability of female *Culex pipiens*, by calculating of LD_{25},LD_{50} , LD_{75} and LD_{90} that equal 20,35,60 and 95 Gy, respectively. The usage of gamma rays as sterile insect technique may succeed in the control of female *Culex pipiens*, but on the other hand may lead to transmission of HCV from infected person to another, because gamma rays according to the previous study affecting all the insect structure and function which maybe leads to the level of HCV inside the irradiated *Culex pipiens* post feeding on the infected person more than the non- irradiated insects.

The major radiation effects on insects are reported by El-Naggar, (2009) are aggregate formation, changes in serological characteristics, decrease or arrest of proteins synthesis, partial or total loss of function, changes of chemical structure, and disturbances in transamination process. Also, radiation is the diminished synthesis of globin and haem, decrease antibody formation, changes in normal Albumin/ Globulin ratio with the increase in globulin and decrease of albumin, whereas, also gamma radiation reduced absorption of fats and fat-soluble vitamins. In addition, oxidized of unsaturated fatty acids form (lipid peroxides) hydroperoxides (which exhibit radiomimetic properties). Carbohydrate serves as a source of energy and may be converted to fats for storage and to amino acids (Chapman, 1998). The main effects of radiation on carbohydrate appear in that decrease of glucose absorption from intestines, degradation, chain breaks, and depolymerization (loss of ability of polymer formation) of carbohydrate molecules especially polysaccharides. Reducing total carbohydrates induces stress in the insects which in turn reduces some of the vital components in the body. Under such stress conditions, the nutrients get catabolized to meet the high energy demand (Seyoum *et al.*, 2002).

Gabarty *et al.*, *(*2013) reported that gamma irradiation decreases the humoral immune enzyme response of (phenoloxidase, prophenoloxidase, lysozyme) and protein concentration in the 6^{th} instar larvae of *Spodoptera littoralis.* In addition, Exposure to 100 Gy of gamma radiation markedly decreased (THC)/mm3 and the percentage of all cellular immune types (Pl, Gr, Sph, Adip, Cys and Oen) during the course of examination (El-Sonbaty *et al.*, 2016)

The current study involved an experimental investigation of feeding *Culex pipiens* mosquitoes on infected blood through feeder membrane with HCV genotype IV of a known viral concentration of (1.2×10^6 IU/ ml) in blood meal of irradiated females mosquitoes emerged from 60 Gy irradiated pupae and with viral concentration of (1.3×10^6 IU/ ml) in blood meal of non-irradiated one. Mosquitoes were then collected at different intervals post feeding and subjected to real time-PCR analyses. Results showed that all mosquitos' mouth parts collected at time zero

(directly after feeding), 30 min and 60 min post-feeding were positive for HCV genome using real time-PCR analysis.

Our results were in harmony with Hassan *et al.* (2003) who reported that HCV-RNA was detected in heads of symbiotic females at 3hr post feeding and in aposymbiotic ones the virus was detected afterward in head and guts. despite of the presence of some variations in the length of the period at which the virus can be recovered from mosquitoes after feeding on infected blood. The procedure implemented can be described as the variation in the longer persistence of HCV shown (3 h) in comparison to that reported in our study (1 h) may be attributed to many factors including the viral genotype, temperature and the environmental factors of the insect breeding but not limited to the number. Also, our results were similar to Tarish *et al.* (2014) who investigated the infectivity titer through mouthparts of female mosquitoes, previously fed on HCV infected blood, after maintaining their feed via Para film 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.

However, the present results showed a negative correlation between the HCV titer and time. Although the titer decreases when the time increased, it may contribute and raise the probability of mechanical transmission of HCV. This conclusion was in accordance to Kamal (2008) who reported that one viral particle perhaps sufficient to induce infection through parenteral route.

Our results also showed evaluation the infectivity titer through mid-gut in irradiated and non-irradiated mosquitoes. All experiments showed a decrease in the virus titer by the time. Despite of the titer percentage of HCV decreased, the irradiated *C. pipiens* mosquitoes are still precursor to the virus, this may due to the effect of radiation on mid- gut flora and the biological system of insects (El-Naggar, 2009.; Chapman, 1998.; Seyoum *et al.,* 2002 Gabarty *et al.,* 2013 and El-Sonbaty *et al.,* 2016).

Our results also evaluated the infectivity titer through salivary glands in irradiated and non-irradiated mosquitoes at 5 , 6,11,12 and 13 days post feeding. The experiment showed negative results for HCV RNA. These results were similar to Chang *et al*. (2001) who prepared the group of *C. quinquefasciatus* mosquitoes infected 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* showed negative results. These results indicated that the biological transmission of HCV in irradiated *Culex* mosquitoes doesn't occur.

The usage of gamma rays as sterile insect technique may succeed in the control of *Culex pipiens,* but an increase of the infection rate percentage in the mid gut of irradiated females makes us recommended the usage of male sterile insect technique Hassan *et al*. (2017) in the control of *C. pipiens* mosquitoes is more effective and safer than female sterile insect technique. Thus, in accordance with Robinson and Franz (2000) reported that the release of sterile females is not acceptable for those species where the females are vectors of disease and/or cause the biting nuisance.

In conclusion, by comparing the irradiated female with non- irradiated female in their ability for the transmission of HCV, we find that mechanical transmission is possible in irradiated and non – irradiated females but biological transmission did not occur in the two cases. the usage of male sterile insect technique in the control of *C. pipiens* mosquitoes is more effective and safer than female sterile insect technique.

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