Original Article

Evaluation of Isotope ^{32}P Method to Mark *Culex pipiens* (Diptera: Culicidae) in a Laboratory

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Abstract

Background: The aim of the current study was to develop a marking technique as an internal marker to mark post blood meal mosquitoes by using stable phosphate isotope ^{32}P and determine the optimal concentration of it.

Methods: An isotonic physiological saline solution, containing different concentration of radioactive isotope ^{32}P-labeled disodium phosphate ($Na_2H^{32}PO4$) was injected into rabbits via the jugular vein in the laboratory. Emerged *Cx. pipiens* were marked after feeding on rabbit. At the same time, the labeled conditions of emerged *Cx. pipiens* were also measured by placing feces of No. 6 rabbit into containers with mosquito larvae and pupae inside.

Results: According to the label condition of *Cx. pipiens* after taking blood and the effect of different dosage $Na_2H^{32}PO_4$ on rabbit health, the optimal concentration of radioactive isotope was determined, that is, 0.1211 mCi/kg. By placing feces of No. 6 rabbit into containers with mosquito larvae and pupae inside, the emerged mosquitoes were also labeled. Therefore, feeding mosquitoes on the animal injected with radioactive $Na_2H^{32}PO_4$ was more practical for detecting and tracing mosquitoes.

Conclusion: The method was less time-consuming, more sensitive and safer. This marking method will facilitate post-bloodmeal studies of mosquitoes and other blood-sucking insects.

Keywords: Radioactive isotope, Mark, *Culex pipiens*, Rabbit

Introduction

Mosquitoes, the most important group of nuisance pest insects, due to their diversity and abundance, demonstrated vector competence and frequent infection in nature, they are regarded as one of the most important vectors of diseases (Sardelis et al. 2002, Molaei et al. 2006, Burkett-Cadena et al. 2008a, b). Tracking the movement of mosquitoes in their natural habitat is critically important for understanding their basic biology, demography, ethology and vector-borne disease control as well as prevention. A reliable method for marking is critical important to study mosquito behavioral characteristics.

Animal marking have been used since 218 BC when Fisher and Peterson used banding to distinguish ownership of birds (Fisher and Peterson 1964). Unfortunately, for marking insects, most marking techniques for vertebrate, such as bands, brands, tattoos, tags, notches, paints are not practical because they are tedious, time-consuming, heavy and costly (Southwood 1978, Basavaraju et al. 1998). Insect marking for scientific studies dates back to 1920, since then, a variety of marking techniques, paints, dyes etc. were used to in studies of insect population dynamics (Geiger et al. 1919, Dudley and Searles 1923).

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Methods to mark mosquitoes have included dyes (Welch et al. 2006, Midega et al. 2007), paints (Trpis and Hausermann 1986, Niebylski and Meek 1989, Service 1993, Bellini et al. 2010, Ciota et al. 2012, Liu et al. 2012, Verhulst et al. 2013), dusts (Reisen et al. 1978, Reisen et al. 1992, Russell et al. 2005), trace elements (Anderson et al. 1990, Holbrook et al. 1991, Solberg et al. 1999, Wilkins et al. 2007), and radioactive isotopes (Jenkins 1949, Abdel-Malek 1966, Lindquist et al. 1967, Hood-Nowotny et al. 2006, Hamer et al. 2012). However, for study on the behavioral characteristics of post bloodmeal, existing techniques tend to be labor intensive, as they require rearing mosquitoes, marking them in large quantities, and then inspecting large numbers of individuals to detect recaptures (Walker et al. 1987). Furthermore, compared with natural populations, rearing mosquitoes, marking them in large quantities by using artificial methods, and releasing them may change their behavior (Reisen et al. 2003, Silver 2008). For study the behavior characteristics after blood meal, these methods are not ideal. However, the problem is how to mark breeding mosquitoes without inhibiting their normal biology, and with long-term retention after blood meal, it is still bothering most biological scientists. Until now, only Zhang et al. (2014) marked adult mosquitoes by feeding them on cow injected with isotope ^{32}P and subsequent ecological investigations. In some studies, successfully labeled mosquitoes by feeding them on radioactive animal blood (Hassett and Jenkins 1951), use of large bait animals for marking wild population of adult *Anophels aquasalis* injected with a dose of 1.7 curies of ^{32}P (Bruce-Chwatt 1956). However, such high dosage would be dangerous to the animal (Winteringham London meeting, 1953).

The aim of the current study was to develop a marking technique as an internal marker to mark post blood meal mosquitoes by using stable phosphate isotope ^{32}P and determine the optimal concentration of it.

Injection of rabbits with $Na_2HP^{32}O_4$ and blood feeding of *Culex pipiens*

Before experimenting, 6 healthy rabbits (2 kg) were selected and physically examined by veterinarian. The injection method of normal saline solution to the rabbits was according to Smith et al. (1951), i e. An isotonic buffered saline solution, containing different concentration of $Na_2HP^{32}O_4$, was injected intravenously of rabbits. The dosage for No. 1, 2 rabbits, No. 3, 4 rabbits, No. 5 rabbit and No. 6 were 0.2 mCi, 0.4 mCi, 0.8 mCi and 1.7 mCi, respectively. For the negative control, 1 healthy rabbits was injected with isotonic buffered saline solution without $Na_2HP^{32}O_4$.Then 20 to 50 emerged female *Cx. pipiens* fed on these rabbits at 6, 12, 24, 48, 72 h and 120 h after injected with $Na_2HP^{32}O_4$. At the same time, 0.2 ml of blood was extracted from rabbits, as well as at 16th and 32nd days for radioactivity level measure. Every batch of *Cx. pipiens*' radioactivity levels were measured at 2 h, 6 h, 12 h, 24 h, 48 h, 72 h and 120 h after blood meal.

Measuring methods

The measurement of radioactivity was conducted using a Liquid Scintillation Counters (Model YSJ-76). Before injection of ^{32}P to rabbits, to normalize background radiation, radioactivity level of 30 emerged adult *Cx. pipiens* reared in the lab was measured. Mosquitoes tested were anesthesia with ether, placed in a β bell counter tube of the vitriol chambers for 1 minute, as for the rabbits blood test, 0.2 ml of blood was dipped on the paper in the tinfoil sample plates, counts that exceeding 50% of the background was as the standard that were labeled.

Placing feces of No. 6 Rabbit in tap water to mark mosquitoes

At the 2nd day after No. 6 rabbit was in-

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jected with $Na_2H^{32}PO_4$, up to 5 g feces was placed into a container with 300 ml of tap water and *Cx. pipiens*' larvae and pupae inside. As the negative control, placed 5 g feces of the rabbit without injected $Na_2HP^{32}O_4$ into the water. Radioactivity levels of emerged mosquitoes were measured.

Ethics clearance

The experimental project was reviewed and approved by the Ethical Committee of Shandong Academy of Medical Sciences (Jinan, Shandong). Urine and feces of the rabbits were collected and sent to the Institute of Radiation Medicine, Shandong Academy of Medical Sciences for appropriate processing to prevent spread of the isotope. The half-life of ^{32}P was 14.3 d.

Results

Conditions of mosquitoes radioactively labeled after blood feeding

Radioactivity level of emerged adult *Cx. pipiens* and rabbits blood was measured, the background was determined as was 12–13 counts per minute (CPM), i e counts that exceeded 50% of background (>20 cpm) were considered positive.

Within 5 days after No. 1 to 5 rabbits were injected with $Na_2H^{32}PO_4$, 579 female *Cx. pipiens* fed on the rabbits, among the 222 mosquitoes blood feeding, except one mosquito was fewer than 20 CPM, the others 221 mosquitoes were labeled no matter how much dosage of $Na_2H^{32}PO_4$ was injected. The more dosage the rabbits injected with $Na_2H^{32}PO_4$, the higher radioactive levels of *Cx. pipiens* after blood feeding. The radioactive levels started to decrease from the 48 hours (2nd day) after injection. Compared with radioactive level at 48 h, though the radioactive level of labeled mosquitoes was higher than background at 72 h, 96h and 120 h, radioactive level decreased to a lower level (Figs. 1, 2, 3).

Radioactivity levels of rabbit blood after injection of $Na_2HP^{32}O_4$

The radioactive level decreased very fast in the blood of rabbits. At 6 hour the radioactive level was 100%, the radioactive level decreased 26–48%, 60–75% and 95–97% at the 2nd, the 5th and the 32nd day, respectively (Table 1).

Effect of $Na_2H^{32}PO4$ on rabbit health

No. 1 to 4 rabbits showed good health, have no change on body temperature and body weight. On the 2nd day, No. 5 rabbit abortion, and gave birth to 2 died bunnies, the radioactive level was 15 mR / h at 5 cm away from these died bunnies. The No. 5 rabbit presented no abnormalities and was dissected at the 143rd day after injected with $Na_2H^{32}PO4$, no pathological changes were found in the internal organs. The radioactivity level of liver was measured, which decreased to similar level of background. However, No. 6 rabbit showed diarrhea, appetite loss, weight loss and other symptoms. At the 31st days, and no special lesions were found in internal organs after dissection. It still presented a higher radioactivity level in liver, spleen, intestine, heart, lung, kidney, muscle etc (Table 2).

Conditions of mosquitoes radioactively labeled after placing feces in the container

At the 2nd day after No. 6 rabbit was injected with $Na_2H^{32}PO_4$. After 5 g feces of No. 6 rabbit was placed into a container, at the 2nd and 7th day, there were total 39 adult *Cx. pipiens* mosquitoes emerged, among them, 22 (56.5%) were marked, their CPM was 21 875, the average was 323.

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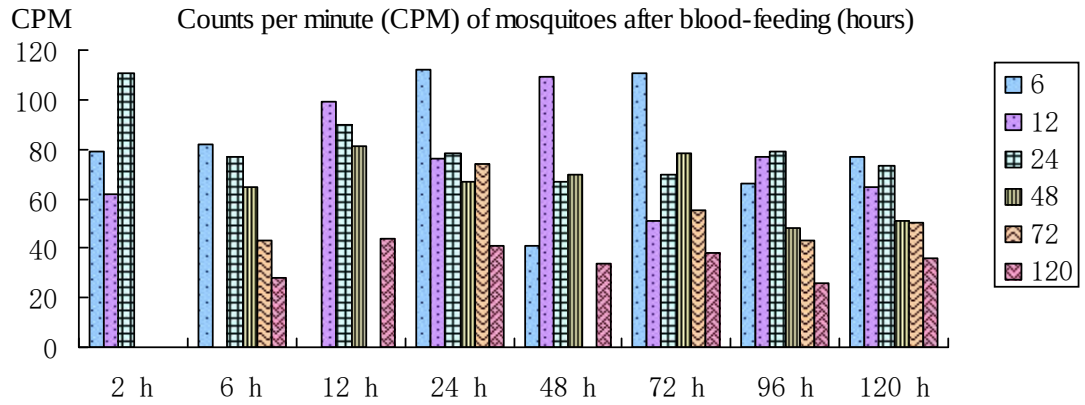

Fig. 1. Levels of radioactivity of *Culex pipiens* after fed on No. 1, 2 rabbits after injection of ^{32}P (0.2 mCi)
Legend 6, 12 , 24, 48, 72, 120: mosquito fed time after injection ^{32}P into rabbit (hours)

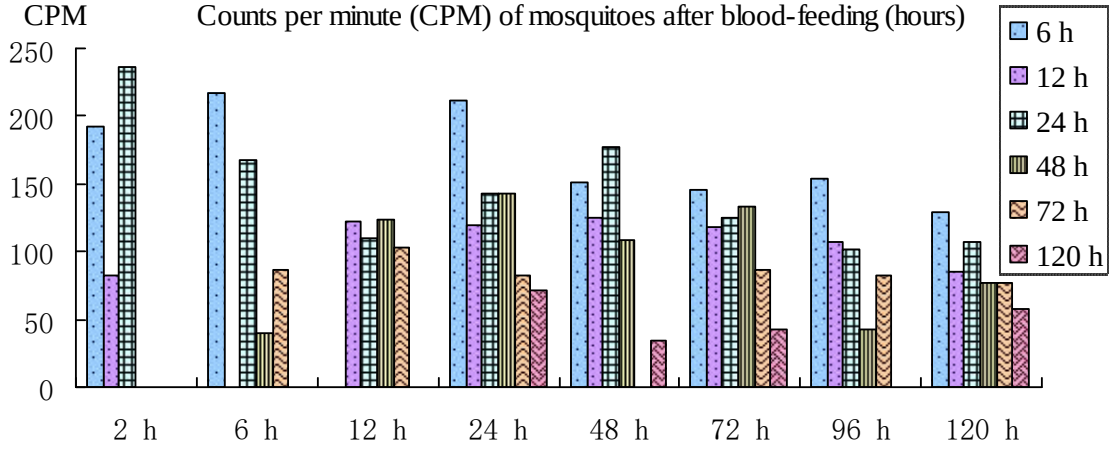

Fig. 2. Levels of radioactivity of *Culex pipiens* after fed on No. 3, 4 rabbits after injection of ^{32}P (0.4 mCi)
Legend 6, 12, 24, 48, 72, 120: mosquito fed time after injection ^{32}P into rabbit (hours)

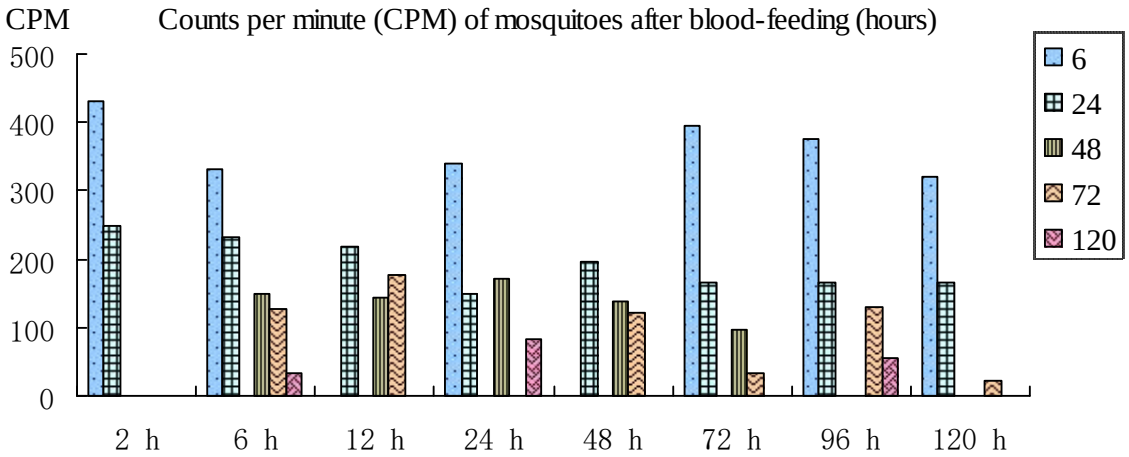

Fig. 3. Levels of radioactivity of *Culex pipiens* after fed on No. 5 rabbits after injection of ^{32}P (0.8 mCi)
Legend 6, 24, 48, 72, 120: mosquito fed time after injection ^{32}P into rabbit (hours)

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Table 1. Radioactivity levels of rabbit blood after injection of ^{32}P (1.7 mCi) at different time

Rabbit No.	Dosage (mCi)	Radioactivity level of rabbit blood after injection of ^{32}P														
		6h	12h		24h		2 days		3 days		5 days		16 days		32 days	
		CPM	CPM	Decrease%	CPM	Decrease%	CPM	Decrease%	CPM	Decrease%	CPM	Decrease%	CPM	Decrease%	CPM	Decrease%
1, 2	0.2	2709	1922	29	1701	37	1413	48	992	63	681	75	226	92	84	97
3, 4	0.4	4155	3383	19	2926	30	2383	43	1820	56	1199	71	317	92	120	97
5	0.8	5701	5189	9	5290	7	4234	26	3681	35	2300	60	783	86	261	95
6	1.7	9284	-	-	-	-	-	-	-	-	-	-	1,426	85	-	-

CPM = pulse per minute,
- Do not measured

Table 2. Radioactivity levels in No. 6 rabbit tissue after injection of ^{32}P (1.7 mCi) 31 days

Tissue	Weight (mg)	CPM	CPM per tissue
Liver	275	5,263	19,138
Muscle	295	5,586	19,000
Spleen	197	3,118	15,827
Heart	336	5,170	15,387
Small intestine	205	2,419	11,800
Lung	270	2,685	9,944
Kidney	306	2,628	8,588

Background radiation= 22 CPM

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Discussion

We could see that the more dosage the rabbits injected with $Na_2H^{32}PO_4$, the higher radioactive levels of *Cx. pipiens* after blood feeding. The radioactive levels started to decrease from the 48 hours (2^{nd} day) after injection. Compared with radioactive level at 48 h, though the radioactive level of labeled mosquitoes was higher than background at 72 h, 96 h and 120 h, radioactive level decreased to a lower level. The more injection of $Na_2H^{32}PO_4$ into rabbits, the higher radioactivity level in the blood and the slower decrease, was also consistent with the labeling condition of *Cx. pipiens* (Figs. 1, 2, 3).

No. 1 to 4 rabbits showed good health, but as for the No. 5 rabbit injected with 0.8 mCi of $Na_2H^{32}PO_4$, on the 2^{nd} day, abortion, and gave birth to 2 died bunnies. At the 143^{rd} day, the No. 5 rabbit presented no abnormalities, no pathological changes were found in the internal organs. The radioactivity level of liver was similar to background. However, No. 6 rabbit injected with 1.7 mCi of $Na_2H^{32}PO_4$ showed diarrhea, appetite loss, weight loss and other symptoms. At the 31^{st} days, it still presented a higher radioactivity level in liver, spleen, intestine, heart, lung, kidney, muscle etc. (Table 2). Therefore, the appropriate concentration for not only marking mosquitoes but also no harm to rabbits was not more than 0.4 mCi.

Stable isotopes occur naturally in the environment, are safe and non-invasive, pose no health or environmental risks (Hood-Nowotny and Knols 2007). In medical research, most stable isotopes are non-toxic and are routinely used for mosquito feeding trials, in which human adults are 'labelled up' through supplementary feeding with stable isotopes, may be useful for host seeking behavior and repellent testing, etc., in 'real' environments. Several tracers were studied, such as ^{60}Co, ^{89}Sr, ^{65}Zn, ^{131}I, ^{45}Ca and ^{32}P, whereas ^{32}P was the most applied radioisotope for tagging due to its short half-life, safety, activity and easy of detection (O'Brien and Wolfe 1964). One of the earliest examples of using inorganic ^{32}P labelled *Ae. aegypti* mosquitoes was reported by Hasset and Jenkins (1949). Hasset and Jenkins (1951) also performed a detailed study of the conditions affecting mosquitoes labelled with ^{32}P and compared stages, ^{32}P concentrations and age. The filarial larvae, *Setaria digitata* Linstow was marked after adult mosquitoes (*Armigerea obturbans* Walker) fed on cows or men infected with microfilaria, that larvae of mosquitoes were reared in water containing 1 μc of ^{32}P/mL (Dissanaike et al. 1957).

Toxicity to the insect was also a serious problem to be considered in many studies (Quarterman et al. 1955). The radioisotopes ^{45}Ca and ^{131}I were very toxic when fed to adult houseflies at 1 μc/mL of milk, whereas ^{32}P was satisfactory (Quarterman et al. 1954). By using ^{15}N-labeled potassium nitrate and ^{13}C-labeled glucose to mark larval mosquitoes, there were no consistent effects of isotopic enrichment on immature mosquito survival or adult mosquito body size (Hamer et al. 2012).

Although fluorescent dyes or powders are also suitable for marking mosquitoes (Takken et al. 1998, McCall et al. 2001, Pates 2002, Lapointe 2008, Baber et al. 2010, Bellini et al. 2010), and no effect of these dyes and powders on performance of ant (*Pogonomyrmex owyheei*), mountain pine beetle (*Dendroctonus ponderosae*), grasshopper (*Melanoplus* spp.), *Ae. aegypti*, *Anopheles sinensis* in some studies (Porter and Jorgenson 1980, Linton et al. 1987, McMullen et al. 1988, Narisu et al. 1999, Liu et al. 2012, Valerio et al. 2012). Others have found a reduced longevity, behavioural response or survival of opine parasitoids (*Diachasmimorpha* spp.), weevils, *Ae. aegypti*, Asian citrus psyllid (*Diaphorina citri*) and codling moths (*Laspeyre-*

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sia pomonella) (Sheppard et al. 1969, Moffitt and Albano 1972, Reinecke 1990, Messing et al. 1993, Nakata 2008, Verhulst et al. 2013). As for the fluorescent marker, be caused of the restrictions of the retention, most studies researches primarily focus on dispersal of nulliparous female mosquitoes during the initial host-seeking event and sometimes a second host-seeking event (Hamer et al. 2012).

As for trace element, mosquitoes were successfully marked after feeding on hosts injected with trace element Rb and Cs (Anderson et al. 1990, Solberg et al. 1999). Marking with trace elements has many advantages over other insect-marking procedures. They are not radioactive, safe for workers and for the environment. As for insects marked with an element, no tags, paints, dyes, dusts, or visible marks were found left to alter insects behavior or interactions with other insects (Hagler and Jackson 2001).

However, a limitation to use of trace elements as insect markers in large fields is that the detection of elements can be difficult, expensive, and time-consuming, requires technical expertise and expensive detection equipment (Akey and Burns 1991). Some trace elements are not retained very well in certain insect species, for example, Rb could be detected for only 2–6 days after marking aphids (Guillebeau et al. 1993) and adult *Lygus lineolaris* (Fleischer et al. 1986). High concentrations of trace elements can adversely affect development, survival, increased adult deformity, reduced pupation, eclosion, egg production and fecundity of certain insects (Stimmann et al. 1973, Hayes 1989, Knight et al. 1989, Van Steenwyk et al. 1992).

Naturally, occurring stable isotope markers are useful, as they do not require the premarking of individuals (Hood-Nowotny and Knols 2007). Labelling a distinct portion of an ecosystem with stable isotopes is a useful, minimally invasive method to study insect dispersal from an ecophysiological perspective (Macneale et al. 2004, 2005). Stable isotopes occur naturally in the environment, unlike painting, dusting, etc., stable isotope methods are non-invasive and samples require only minimal preparation following collection, which makes the cost of the process as a completely comparable to methods such as polymerase chain reaction (Hood-Nowotny and Knols 2007). Other advantages are the analysis costs (depending on the isotope and the matrix, the cost per sample may range from US$ 5–100.00), shipping stable isotope samples is simple, safe and inexpensive (IAEA 2009). It would cost between $150–250 to label 1 000 000 *Anopheles* mosquitoes with ^{13}C-labelled glucose in the larval stages (Hood-Nowotny et al. 2006). It is possible to trace the fate of labelled sperm into female spermatheca in studies of male mosquitoes labelled with ^{13}C (Helinski et al. 2007). These stable isotope markers meet the usual criteria for use in insect studies: retention, no effect on behavior, durability, easily applied, clearly identifiable, and not expensive (Hagler and Jackson 2001).

A labelled blood source also provides an easily identifiable point source for post feeding dispersal studies. Tracing of labelled blood to determine resource allocation to the eggs or other tissues could also provide useful physiological information. It was possible to mark large numbers of mosquitoes by providing blood meals from a host fed injected with a stable isotope substance.

Conclusion

$Na_2H^{32}PO_4$ was injected via the jugular of rabbit vein; these rabbits were bitten by emerged female *Cx. pipiens*, so mosquitoes were successfully marked. The appropriate dosage (not only can label mosquito but also have no ill effects on rabbits) of $Na_2H^{32}PO_4$ was 0.1 mCi/kg. The emerged adult *Cx. pipiens* mosquitoes were also can be marked by placing rabbit feces or $Na_2H^{32}PO_4$ into

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container. The technique was less time-consuming, more sensitive, and safer, can be used to study post-blood meal dispersal of mosquitoes and other blood-sucking insects. The short half-life of 14 days of ^{32}P, however, was a disadvantage in studies where prolonged observations were necessary.

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