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Investigation of *Aedes aegypti*'s resistance toward insecticides exposure using Markov modeling

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Abstract
Aedes aegypti is the vector of dengue infection. One of the methods to control the mosquitoes is the uses of temephos. However, continuous exposure of insecticides can cause resistance toward temephos. This research studied the *Aedes aegypti*'s behavior changes and its resistance toward continuous exposure of temephos. Three sample groups of *Aedes aegypti* from field (endemic and non-endemic) and from laboratory were collected. Larvae used in this research are 80 from field and 80 from laboratory. The samples are divided into eight groups and tested its resistance towards sublethal doses of temephos (0.00375 and 0.005 ppm) and modeled using Markov modeling. Mosquitoes group which has the most severe resistance is the group from endemic area of 4th instar which is given by 0.00375 ppm of temephos with the life time over 463.50 hours. While mosquitoes group which has the least resistance is the group from laboratory of 3rd instar which is given by 0.005 ppm with life time over 5.28 hours. Different origin, insecticide doses, and instar level can cause different effect on the mosquito's resistance.

Keywords: *Aedes aegypti*, insecticide, Markov modeling, resistance, temephos

1. Introduction

Dengue infection is one of the most major public health concerns in Indonesia. Dengue vaccine has not been distributed widely in Indonesia, so the main way to prevent dengue transmission is by controlling vector *Aedes aegypti* (*Ae. aegypti*). *Ae. aegypti* control in Indonesia focused on eliminating breeding site, human protection, and chemical insecticide used [1].

Two classes of insecticide to prevent dengue transmission were pyrethroids and organophosphate. Organophosphates such as malathion and pyrethroids used to control adult mosquitoes. Another type of organophosphate was temephos that used as a larvacide [2]. Organophosphate insecticides (temephos) had been used since early 1970 as a larvacide. Indonesian Ministry of Health data shows that Dengue haemorrhagic fever cases gradually decrease from 2013 until 2015 [3]. However, even Indonesia was one of the major areas for arthropord borne diseases such as *Ae. aegypti*, but reports on the effectiveness of temephos to control those population in Indonesia were limited.

Resistance of temephos had been reported in West Java and Central Java. This report shows that larval insecticide resistance was low to moderate [4]. Therefore it is imperative to have presupposition for the *Ae. aegypti* larvae change after exposed with temephos. Stress that caused by sublethal dose insecticides can enhancer mutation rates of insects [5]. The survivors caused by sublethal mutation were likely more than caused by normal mutation [6]. Sublethal dose can also make changes in mosquitoes behavior such as reduce larvae swimming speed and wriggling movement [7]. In this paper an investigation of *Ae. aegypti*'s resistance to temephos was conducted using Markov modeling. *Ae. aegypti*'s behavior changes toward insecticide exposure are not a definite event. Otherwise, it is happened in a random way so that the Markov modeling can be used other than deterministic model. Statistical analysis which often used in medical experiments, such as t-test, z-test, ANOVA, or other nonparametric tests (that are comparing mean and variance of two or more populations through hypotheses testing), are not suitable for this experiment because they cannot identify mosquitoes behavior changes over time. This model works by analyzing *Ae. aegypti*'s behavior changes which are divided into three states; "healthy" state, "fainted" state, and "die" state. Transition probability of each state was calculated and used to determine resistance probability of the mosquitoes.

Furthermore, mean time needed by the mosquitoes to stay in healthy or fainted state before die is also calculated. Those two measures are used to determine resistance level of *Ae. aegypti* toward the temephos exposure which are used in this research.

2. Materials and Methods

2.1 Mosquitoes

Three sample groups of *Ae. aegypti* were tested in these study. They were collected from two different places of village in Yogyakarta; endemic and non-endemic village and the last sample group as a control was from the laboratory. To obtain the entomological collection, we conduct verbal permission from householders to put down ovitraps. Ovitraps were placed inside houses and in backyard for 200 houses. The houses were located in two different areas of the city which were selected due to its historical of low transmission (non-endemic) and high transmission (endemic). The samples used for this study consist of 80 larvae from field (endemic and non-endemic village) and 80 larvae from laboratory. The samples of each origin are divided into 4 groups (2 groups used 3rd Instar and the other 2 groups used 4th Instar), each consist of 20 larvae, so that there are 8 sample groups in total. Each group will be given by different exposure of sublethal dose of insecticide. The detail of the sample group's division is shown in Table 1.

Table 1: Mosquito's Sample Group Division

Sample group	Insecticide Exposure	Origin	Instar
E-1	0.00375 ppm	Endemic Area	3rd Instar
E-2			4th Instar
L-3		Laboratory	3rd Instar
L-4			4th Instar
NE-5	0.005 ppm	Non Endemic Area	3rd Instar
NE-6			4th Instar
L-7		Laboratory	3rd Instar
L-8			4th Instar

Eggs collection was taken to the insectaries after seven days. Positive traps from all sample groups were reared in laboratory of parasitology in Faculty of Medicine Universitas Islam Indonesia at temperature 25±5 ºC, 78% humidity, and photoperiod 12 hours day/night before used. The eggs were hatched and offspring were reared.

Sample groups, especially from field (endemic and non-endemic area), were only divided into two variations of doses because the eggs were not hatched during rearing process in laboratory. During the research, the sample groups from endemic and non-endemic area were also given by 0.005 ppm dose and 0.00375 ppm respectively, but the result showed that most of the larvae were died before the observation time done. So we exclude the two groups from the analysis.

2.2 Inseticides susceptibility test

This study used Abate 1% (temephos) from BASF. Each bioassay for every sample grop consists of 20 larvae. Each sample group used 3rd and 4th instar of *Ae. aegypti.*

2.3 Resistance assay

Before examination was conducted, we used WHO standard method to find out the resistance level of each sample group [8]. After the resistance status known, we used the sublethal dose to examine the change of resistance of *Ae. aegypti* in 24 hours. From the preliminary study we find the sublethal dose for all sample groups were 0.00375 ppm and 0.005 ppm. The determination of the two doses is based on the fact of the previous study that the larvae will all live if the doses are below 0.00375 ppm. While for the doses above 0.005 ppm, most of the larvae died. Behavior changes of every sample group were noted every hour until 24 hours.

2.4 Data analysis

Bioassay data were analyzed using Markov modeling to determine the resistance level of *Ae. aegypti*. It is divided into four levels; susceptible (not resistance), minor resistance, medium resistance, and severe resistance.

3. Markov Modeling

Assume that there are three possible states of *Ae. aegypti*'s behavior changes after a given exposure of insecticide, i.e. "healthy" (S_0), "fainted" (S_1), and "die" (S_2).

Table 2: Mosquito Resistance State

Code	States	Description
0	Healthy	Mosquitoes actively moving up and down when given stimulants
1	Fainted	Mosquitoes less active and pale when given stimulants
2	Death	Mosquitoes do not move when given stimulants

The pattern of their behavior changes is described by Fig. 1.

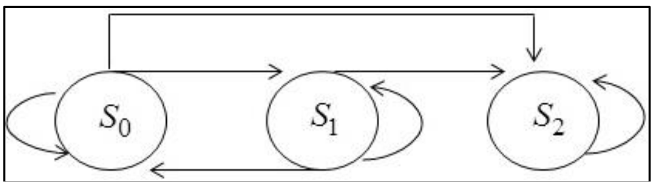

Fig 1: Pattern of *Aedes aegypti*'s Behavior Changes.

Transition probability of mosquito's behavior changes can be determined by evaluating the probability of mosquito will be in state S_j, $j = 0,1,2$ at time $t+1$, given the mosquito is in state S_i, $i = 0,1,2$ at time t, i.e.

$$P(X_{t+1} = S_j \mid X_t = S_i, X_{t-1} = S_{i_{t-2}}, \dots, X_0 = S_{i_0}) = P_{S_i S_j} \quad (1)$$

The transition probability in Eq. (1) can be written in matrix form, notated by P, named as Markov chain, having size 3×3 as follows.

$$P = \begin{pmatrix} P_{S_0 S_0} & P_{S_0 S_1} & P_{S_0 S_2} \\ P_{S_1 S_0} & P_{S_1 S_1} & P_{S_1 S_2} \\ P_{S_2 S_0} & P_{S_2 S_1} & P_{S_2 S_2} \end{pmatrix} \quad (2)$$

Based on their behavior pattern, the resistance categories of *Ae. aegypti* are given by

Table 3: Resistance Categories of *Aedes aegypti*

Resistance			Susceptible		
S_0	→	S_0	S_0	→	S_2
S_0	→	S_1	S_1	→	S_2
S_1	→	S_0			
S_1	→	S_1			

Table 3 shows that the mosquitoes are said to have resistance if, within the observation time, they stay healthy or stay fainted and also if their behavior change from healthy to fainted or from fainted to healthy. While the susceptible category is given to the mosquitoes which die during the observation time.

Resistance probability of the mosquito is defined as the average probability that the mosquito is in state S_0 will be in state S_0 or S_1, and the average probability that the mosquito is in state S_1 will be in state S_0 or S_1. Mathematically, the resistance probability of *Ae. aegypti* is defined by

$$P_R = \frac{1}{4}\left(\sum_{i=0}^{1} \sum_{j=0}^{1} P_{S_i S_j} \right) \quad (3)$$

On the contrary, susceptible probability of *Ae. aegypti* is defined as

$$P_S = \frac{1}{2}\left(P_{S_0 S_2} + P_{S_1 S_2} \right) \quad (4)$$

Based on *Ae. aegypti*'s behavior pattern, state S_0 and S_1 are transient states, while state S_2 is an absorbing state. Consider now a finite state Markov chain P_T consist of the probabilities of transient states. The transition matrix can be written in canonical form [9].

$$P = \begin{pmatrix} P_T & R \\ 0 & I \end{pmatrix} \quad (5)$$

For transient state S_0 and S_1, let $t_{S_i S_j}, i, j = 0,1$ denote the mean time spent that Markov chain is in state S_i given that it started in state S_j. Now let

For transient state S_0 and S_1, let $t_{S_i S_j}, i, j = 0,1$ denote the mean time spent that Markov chain is in state S_i given that it started in state S_j. Now let

$$\tau_{S_i S_j} = \begin{cases} 1 & if\ S_i = S_j \\ 0 & otherwise \end{cases}$$

Mean time spent in transient states can be determined by calculating [10].

$$t_{S_i S_j} = \tau_{S_i S_j} + \sum_{k=0}^{1} P_{S_i S_k} t_{S_k S_j} \quad (6)$$

Let T be a matrix consists of $t_{S_i S_j}$, then T can be obtained in matrix form

$$\begin{aligned} T &= I + P_T T \\ &= \left(I - P_T \right)^{-1} \end{aligned} \quad (7)$$

Classification of mosquito's resistance level is defined from its resistance probability (P_R) and mean time spent in transient state (T), that are

Table 4: Mosquito's Resistance Level

Resistance Level	Resistance Probability	T (hours)
Susceptible (Not Resistance)	$0 \le P_R \le 0.7$	$0 \le T \le 3$
Minor Resistance	$0.7 < P_R \le 0.9$	$3 < T \le 12$
Medium Resistance	$0.9 < P_R \le 0.95$	$12 < T \le 24$
Severe Resistance	$0.95 < P_R \le 1$	$T > 24$

Table 4 used as the reference by the researcher to determine *Ae. aegypti*'s resistance level. The two requirements should not be all filled by the mosquitoes, if only one requirement filled by the mosquitoes, then the mosquitoes can be classify into the appropriate resistance level.

Furthermore, the probability that there are n mosquitoes alive after 24 hours can be determined using pure death analysis from continuous time Markov chain method. Assume that there are n mosquitoes in each sample group, death pattern of the mosquitoes with death rate μ is described in Fig. 2

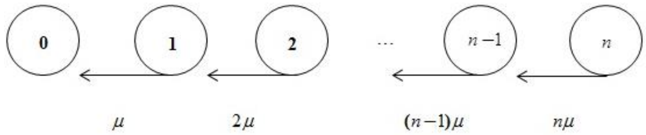

Fig 2: Pattern of Mosquito's Death Rate

Here is the algorithm (1) to calculate the probability

i. Calculate the death rate of each mosquito's sample group, notated as μ

ii. Determine transition rate matrix Q for each sample group

$$Q = \begin{pmatrix} 0 & 0 & \cdots & 0 & 0 \\ \mu & 0 & \cdots & 0 & 0 \\ \vdots & \vdots & \ddots & \vdots & \vdots \\ 0 & 0 & \cdots & (n-1)\mu & 0 \end{pmatrix} \quad (8)$$

iii. Calculate the probability that there are n mosquitoes ($\pi_n(t)$) in the system by solving the global balance equation [11].

$$\frac{d}{dt}\pi(t) = \pi(t)Q \quad (9)$$

which implies

$$\begin{cases} \frac{d}{dt}\pi_n(t) = -n\mu\pi_n(t) & \Rightarrow \pi_n(t) = e^{-n\mu t} \\ \frac{d}{dt}\pi_i(t) = (i+1)\mu\pi_{i+1}(t) - i\mu\pi_i(t) & i = 0,1,\ldots,n-1 \end{cases}$$

And

$$\pi_i(t) = \binom{n}{i}\left(e^{-\mu t}\right)^i\left(1-e^{-\mu t}\right)^{n-i} \quad (10)$$

Plot the graph of $\pi_i(t)$

4. Results and Discussion

Each sample group was observed for 24 hours and its behavior changes were recorded every hour. Based on the results of the records after 24 hours, the number of mosquitoes which were in healthy, fainted, and die state is shown in Fig. 3.

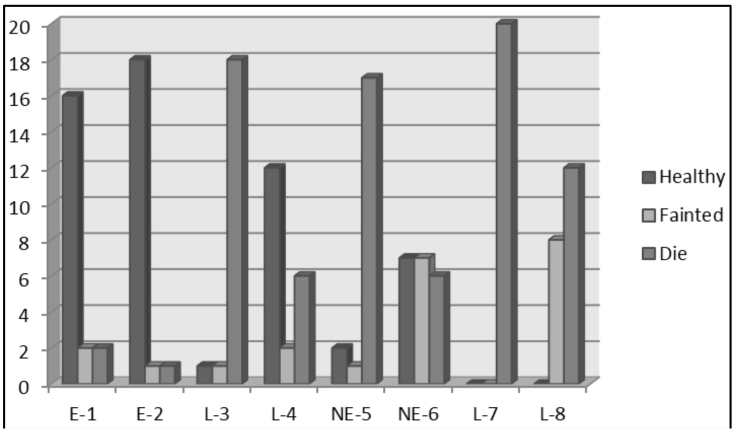

Fig 3: Number of Mosquitoes in Each Sample Group after 24 Hours.

Fig. 3 shows that from the sample used in the research, after 24 hours of observation, the number of mosquitoes that still live at healthy state at most is sample group 2, that is a group of mosquito from field and given by insecticide with concentration of 0.00375 ppm and are in 4th instar. While the number of mosquitoes that still live either in healthy and fainted state at least is sample group 7, that is a group of mosquito that are from laboratory which given by insecticide with concentration of 0.005 ppm and are in 3rd instar.

Using the sample groups, the resistance level of *Ae. aegypti* for each sample group can be obtained by calculating the transition probability of behavior changes of mosquitoes which consist of three states, i.e. S_0, S_1, and S_2. Table 5 gives the result of transition probability matrix which have been calculated using Eq (2).

Table 5: Transition Probability Matrix.

Transition Probability Matrix	
$P_1 = \begin{pmatrix} 0.984694 & 0.015306 & 0 \\ 0.042553 & 0.914894 & 0.042553 \\ 0 & 0 & 1 \end{pmatrix}$	$P_5 = \begin{pmatrix} 0.857143 & 0.092437 & 0.05042 \\ 0 & 0.731707 & 0.268293 \\ 0 & 0 & 1 \end{pmatrix}$
$P_2 = \begin{pmatrix} 0.988739 & 0.009009 & 0.002252 \\ 0.230769 & 0.769231 & 0 \\ 0 & 0 & 1 \end{pmatrix}$	$P_6 = \begin{pmatrix} 0.918831 & 0.064935 & 0.016234 \\ 0.123711 & 0.865979 & 0.010309 \\ 0 & 0 & 1 \end{pmatrix}$

$P_3 = \begin{pmatrix} 0.744681 & 0.244681 & 0.010638 \\ 0.066667 & 0.706667 & 0.226667 \\ 0 & 0 & 1 \end{pmatrix}$	$P_7 = \begin{pmatrix} 0.649123 & 0.263158 & 0.087719 \\ 0 & 0.772727 & 0.227273 \\ 0 & 0 & 1 \end{pmatrix}$
$P_4 = \begin{pmatrix} 0.925 & 0.06875 & 0.00625 \\ 0.140351 & 0.824561 & 0.035088 \\ 0 & 0 & 1 \end{pmatrix}$	$P_8 = \begin{pmatrix} 0.88806 & 0.100746 & 0.011194 \\ 0.090909 & 0.827273 & 0.081818 \\ 0 & 0 & 1 \end{pmatrix}$

Based on Table 5, the resistance and susceptible probability of *Ae.aegypti* were calculated using Eq. (3) and Eq. (4). Comparison of resistance and susceptible probability of each sample group is shown in Fig. 4.

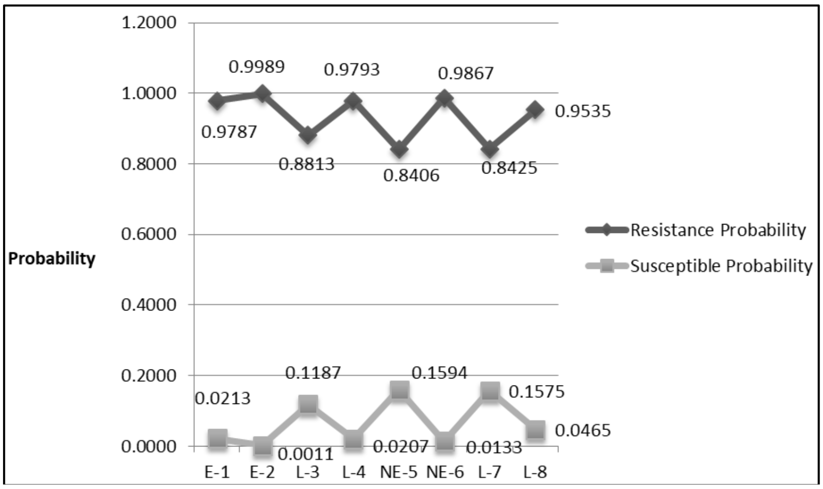

Fig 4: Resistance and Susceptible Probability of *Aedes aegypti*.

Fig. 4 shows that sample group 2 has the highest probability of resistance, while sample group 5 has the smallest probability of resistance. The resistance level for each sample group can be determined by comparing the probability of resistance and susceptible of each sample group and also the mean time spent by each group in healthy and fainted states before die. Furthermore, the mean time spent in transient states (healthy and fainted state) can be calculated using Eq. (7) and the result is shown in Fig. 5

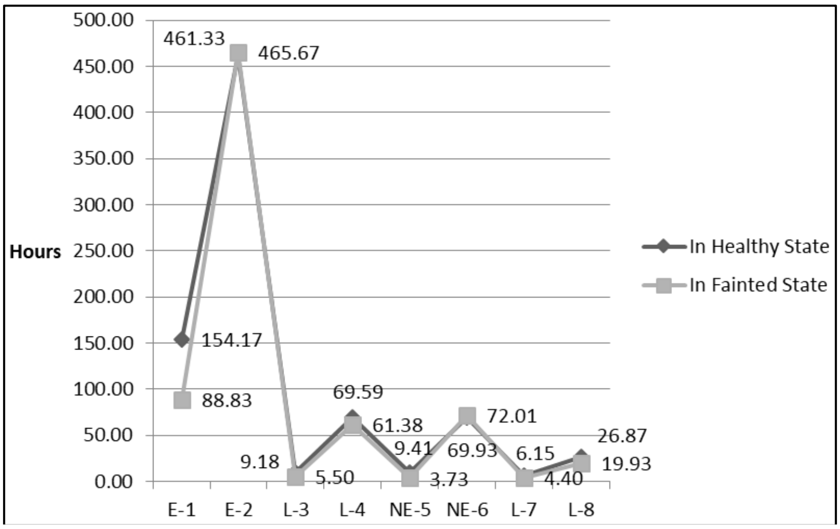

Fig 5: Mean Time Spent in Healthy and Fainted State before Die

Fig. 5 shows the mean time spent by each sample group to stay in healthy and fainted states before die. The mean time indicates the ability of each sample group to survive and resist from the insecticide exposure. Using the results in Fig. 4 and Fig. 5, the resistance level of *Aedes aegypti* for each sample group can be determined based on the classification of mosquito's resistance level from Table 2, and the result is summarized in Table 6.

Table 6: Resistance Level of *Aedes aegypti*

Sample group	Resistance Probability	Mean Time Spent in Transient States T (Hours)			Resistance Level
		In Healthy State	In Fainted State	Mean Time Spent before Die	
E-1	0.9787	154.17	88.83	121.50	Severe Resistance
E-2	0.9989	461.33	465.67	463.50	Severe Resistance
L-3	0.8813	9.18	5.50	7.34	Minor Resistance
L-4	0.9793	69.59	61.38	65.48	Severe Resistance
NE-5	0.8406	9.41	3.73	6.57	Minor Resistance
NE-6	0.9867	69.93	72.01	70.97	Severe Resistance
L-7	0.8425	6.15	4.40	5.28	Minor Resistance
L-8	0.9535	26.87	19.93	23.40	Medium Resistance

From Table 6, it is obtained that there are four sample groups of *Ae. aegypti* having severe resistance, one sample group is medium resistance, and three sample groups are minor resistance. Based on the highest resistance probability and the longest mean time spent in healthy and fainted states before die.

Sample group of E-2 of *Ae. aegypti* having the most severe resistance. *Ae. aegypti* in E-2 was came from endemic area in Yogyakarta. Severe resistance in sample E-2 can be happened because there are mutation processes at genes which are functioned to neutralize the insecticide inside the mosquito's body [12]. Whereas *Ae. aegypti* in L-7 are more susceptible than the other sample groups because it came from laboratory. Mosquito's laboratory is a laboratory control mosquito which is not exposed by insecticide so that it is estimated that there are no mutation yet toward insecticide.

Table 6 also shows that sample group of L-7 having the least resistance because it has the least resistance probability and the fastest mean time spent in healthy and fainted states before die. Observation in this mosquito shows that the temephos exposure on the sublethal doses causes decrease on the mosquito's swimming ability and wriggling movement [7]. Insecticide exposure given to the mosquitoes also showed that in the beginning of the exposure there is a process of adaptation which is indicated by the response of agitation in the form of increased activity due to irritation of the process which was followed by a slowdown in the movement as a result of intoxication to insecticides [13].

The results above show that mosquitoes from laboratory (L-4) also have severe resistance. It can be happened because mosquitoes from L-4 are in 4th instar which was given by low dose of temephos (0.00375 ppm). In this stadium, they have complete anatomy structure and complete molecular enzyme that can neutralize the insecticide [14]. While mosquitoes in L-8 are also from laboratory that are in 4th instar and given by higher dose than L-4 group. L-8 has medium resistance because they also has complete anatomy structure and complete molecular enzyme but given by high dose (0.005 ppm) so that they cannot neutralize the temephos as good as mosquitoes from L-4 [14].

Mosquitoes which are from endemic and non-endemic area in this study are taken from contiguous areas so it is possible that some mosquitoes from endemic will migrate to non-endemic area. It is one reason why there is possibility that mosquitoes from non-endemic area (NE-6) have severe resistance. If there are resistance mosquitoes in non-endemic area, the resistance gene can be inherited to the next generation so that the population of resistance mosquitoes in non-endemic area can be increased [14].

In addition, the probability that there are n mosquitoes alive after 24 hours is obtained using algorithm (1). The result shows in Fig. 6.

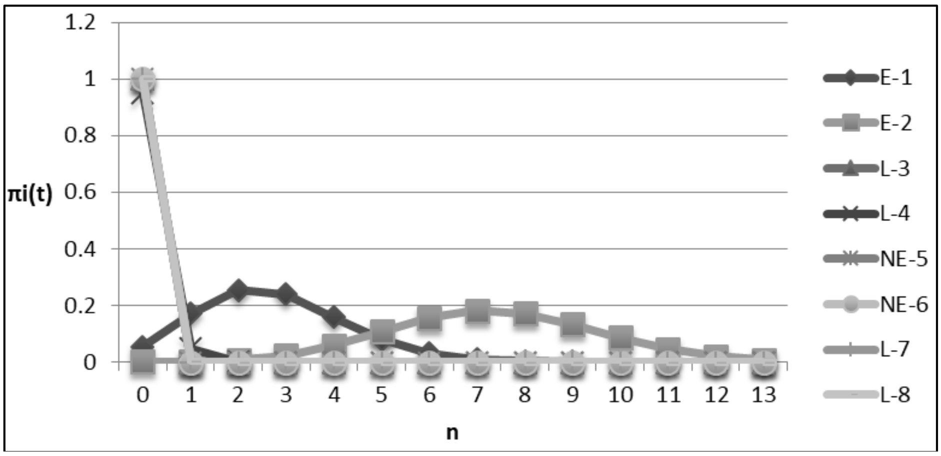

Fig 6: Probability that there are n Mosquitoes after 24 Hours

From Fig. 6 it is known that, except E-1 and E-2, all of sample groups have probability tend to one for no mosquitoes after 24 hours. While in E-1, the probability that there are no mosquitoes after 24 hours is 0.05457 and the probability is 0.000104 for E-2. The probability that there are n mosquitoes (for $n > 0$) is zero for all sample groups except E-1 and E-2. It means that it is almost not possible that all mosquitoes died after 24 hours for E-1 and E-2. This proves that E-1 and E-2 are the mosquito's sample groups which have the most severe level of resistance toward each insecticide exposure compared to the other sample groups.

5. Conclusion

We have modeled resistance probability of *Ae. aegypti* using Markov modeling where the mosquitoes used in this research are divided into 8 sample groups with different origin, sublethal dose of insecticide exposure, and larvae's level. We have calculated the mean time spent in healthy and fainted states of each sample group before die. The two measures are used to classify resistance level of all sample groups. From the analysis we obtain that there are three kinds of resistance level; severe resistance for E-1, E-2, L-4, NE-6, medium resistance for L-8, and minor resistance for L-3, NE-5, L-7. The most severe resistance is the mosquitoes in E-2 which are mosquitoes from endemic area in Yogyakarta and given by the least concentration of temephos (0.00375 ppm). From all of the analysis above, we can conclude that the difference of origin, insecticide exposure, and larvae's level can cause different level of resistance. Generally, the mosquitoes which are from endemic area are usually given by continuous exposure of insecticide that can cause mutation processes at genes. This mutation functioned to neutralize the insecticide inside the mosquito's body so that the mosquitoes from this kind of area have the most severe resistance of insecticide.

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