Larvicidal and ovipositional preference test of copper solution for mosquitoes

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Abstract: In the previous studies, we demonstrated that larvae of *Anopheles stephensi* died within two weeks after the exposure of the larvae to 2.4 ppm of copper solution. The ability of copper to kill mosquito larvae prompted us to examine its applicability to ovitraps. We demonstrated in this study that 10 ppm of copper killed more than 90% of larvae (*Anopheles stephensi*, *Aedes albopictus* and *Culex piopiens pallens*) in 96 hours. At 3.3 ppm, *C. piopiens* was more sensitive than *An. stephensi* and *Ae. albopictus*. Fifty percent of *C. piopiens* larvae died within 24 hours but *An. stephensi* and *Ae. albopictus* survived more than 50% at 48 hours. We also observed that gravid mosquitoes of *An. stephensi*, *Ae. albopictus*, and field-collected *An. sinensis* did not detect nor avoid 10 ppm copper solution for laying eggs under laboratory conditions. These results suggest that copper could be used in ovitraps as a low cost alternative and the eradication of mosquito-borne diseases.

Key words: *Aedes albopictus*, *Anopheles sinensis*, avoidance, copper, larvicides, ovitraps

Introduction

Vector control has proven effectiveness against malaria and other mosquito-borne diseases. Successful malaria eradication programs in several endemic areas have relied predominantly on vector control (Harrison, 1978). For example, the eradication of malaria was achieved in one campaign in Brazil through an integrated approach, which mostly relied on larval control (Killeen et al., 2002). Unfortunately, the effectiveness of vector control has decreased in recent years due to technical and administrative difficulties (Raghavendra et al., 2011).

Several methods have been attempted to control adult mosquitoes. Difficulties have been associated with the control of adult mosquitoes because they move around. In contrast, larvae tend to stay in a limited area. Our recent study on the ability of copper to kill or injure mosquito larvae (Reza et al., 2012) prompted us to utilize it in ovitraps. We examined the abilities of copper sulfate (CuSO₄) and solid copper (copper wire) to kill *An. stephensi* larvae at different concentrations. The result of our first study revealed that copper, in both its solid and liquid forms, was lethal to mosquito larvae at a concentration of 1.2 ppm and impaired the ability of larvae to survive predation by mosquito fish (guppy) at 0.6 ppm. This result encouraged us to apply copper to ovitraps. Many efforts had been made to use ovitraps for surveillance and/or the elimination of adult mosquitoes or mosquito larvae. The findings of one successful test performed in Brazil indicated that this method may be promising for detecting and preventing *Aedes aegypti* outbreaks (Regis et al., 2008). Artificial aquatic habitats have been shown to attract *Anopheles gambiae* s.s., which has led to the construction of gravid traps to control malaria (Dugassa et al., 2012).

Many kinds of lethal ovitraps have been developed to date, including containers filled with larvivorous fish (Pamplona et al., 2009) or insecticides such as imidacloprid (Arreola et al., 2011). A field evaluation in Thailand reported the low efficacy of insecticide-impregnated strips due to fungal contamination (Sithiprasasna et al., 2003). A recent trend is the use of *Bacillus thuringiensis* to kill mosquito larvae. Some researchers reported that *B. thuringiensis* was a potent larvicide in ovitraps (Regis et al., 2008; Carrieri et al., 2009). In spite of its effectiveness, mosquitoes become resistant to *B. thuringiensis* because of spore recycling and the persistence of toxins in the leaf litter (Tetreau et al., 2012). Therefore, an alternative to this larvicide is urgently needed.

We attempted to utilize copper sulfate in ovitraps because it is a cheap alternative to *B. thuringiensis*. Copper sulfate is easily obtained and maintained, and also has bactericidal and fungicidal effects, which is beneficial for bacterial or fungal contamination (Biagi et al., 2014). We also examined the ability of wild mosquitoes (*Anopheles sinensis*) collected in the field to distinguish copper-treated water from regular water. The results of our previous experiment (Reza et al., 2012) prompted us to attempt this method near houses to reduce mosquito outbreaks, especially in areas where mosquito-borne diseases are endemic. This may become an alternative in Integrative Vector Management (IVM) (Beier et al., 2014).
Materials and Methods

Mosquitoes

An. stephensi Liston, 1901, Ae. albopictus (Skuse, 1895), and Cx. pipsens pallens Coquillett, 1898 were reared in our laboratory at room temperature (26°C), 50–70% relative humidity, and a 13 h-light/11 h-dark cycle.

The wild anopheline mosquito, An. sinensis Wiedemann, 1828, was collected in a cattle house near Jichi Medical University using a light adult trap. Since almost all female mosquitoes had fed blood from the cattle, the mosquitoes were allowed to lay eggs in water-containing cups two days later. The purpose of this experiment is to observe the ability for wild mosquitoes to avoid copper containing water.

Observation of mortality of mosquito larvae in copper solutions

Four copper solutions were used in the present study. Copper concentrations were 10, 3.3, 1.1, and 0 ppm. Each solution was allocated to a 12-well cell culture plate with a lid (Corning Incorporated, USA). We initially prepared a solution of 100 mM CuSO₄ by mixing copper sulfate pentahydrate (99.9%) (Wako Pure Chemical Industries, Ltd., Osaka, Japan) with filtered water (Fine Ceramic Filter NGK insulators, Nagoya, Japan). We then prepared the four copper concentrations for the experiments by diluting them from 100 mM CuSO₄ solution and confirmed the ppm level using a Copper Measuring Device (Hanna Instruments, Tokyo, Japan). Then 50 of 1st instar larvae were released in each well and observed every 12 hours for mortality under a microscope.

Choice test of oviposition using blood fed mosquitoes

The mosquitoes, Ae. albopictus and An. stephensi, which were reared in our laboratory, and the wild An. sinensis captured in the field, were used in this experiment. The adult female mosquitoes of Ae. albopictus and An. stephensi had fed on mice three days previously. We prepared four cups containing 70 mL of water in each 30-cm cubic cage. Two cups contained 10 ppm of copper and two cups were copper free (0 ppm). We set the four cups in each corner of the cage. The mosquitoes were allowed to lay eggs in any cup. The cups were removed the next morning and the number of eggs in each cup was counted to examine the ability of mosquitoes to avoid water containing copper ions. This test was performed four times in each species. Proportion of eggs in each cup was presented as a percentage (%).

Statistical analysis

Mosquitoes’ survival rates were presented using Student t-test. Choice tests of oviposition were analyzed using Mann–Whitney U-test.

Results

The survival rates of all three mosquito species were low in 10 ppm of copper. The survival rates of An. stephensi larvae was less than 40% within 24 hours of the treatment, and less than 10% at 36 hours (Fig. 1). Similar results were observed for Ae. albopictus with a 30% survival rates at 24 hours, and 10% at 48 hours (Fig. 2). The survival rate of Cx. pipsens pallens larvae was 10% at 24 hours (Fig. 3). At the lower concentration of copper (3.3 ppm), the tolerance of Cx. pipsens pallens to copper was less than that of An. stephensi and Ae. albopictus. Sixty percent of the Cx. pipsens pallens larvae

Ethics statement

Since we used mice for blood feeding, we received approval from the Institutional Animal Experiment Committee of Jichi Medical University.
died within 24 hours and only 20% survived beyond 96 hours (Fig. 2). In contrast, 50% of the An. stephensi and 40% of the Ae. albopictus larvae survived beyond 96 hours (Figs. 1 and 2). The lowest concentration of copper (1.1 ppm) produced a high survival rate in An. stephensi, only 10% of larvae died after 96 hours (Fig. 1). Sixty percent of Ae. albopictus larvae survived (Fig. 2), while 50% of Cx. pipiens pallens larvae survived at 96 hours (Fig. 3). The survival rate of all three species at 0 ppm was 100% after 96 hours (Figs. 1–3).

The three blood fed mosquito species laid eggs equally in 10 ppm copper cups and copper free cups located in the same mosquito cages (Table 1). The egg counts revealed that no effort had been made by the adult mosquitoes to avoid the copper solution. Eggs were laid randomly in all the containers. All of the larvae in the cups with 10 ppm of copper died within 24 hours after hatching.

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**Discussion**

We utilized copper solutions to determine the most effective dose to kill the larvae of three species of mosquitoes (An. stephensi, Cx. pipiens pallens, and Ae. albopictus) in ovitraps. Copper inflicted high mortality rates (94.6%) at 10 ppm after 96 hours among all three species. The high mortality rate may have been due to the large number of copper molecules that bound with receptors in the larval midgut, because copper was previously shown to damage the perithropic matrix in the midgut (Beaty et al., 2002). A copper concentration of 3.3 ppm killed half of the larvae, but did not achieve an adequate mortality rate after 96 hours. We speculated that not enough copper ions bound with the receptors in the larval midgut at this concentration to produce a high mortality rate in a short period of time; however, further experiments are needed to validate this. The lowest concentration, 1.1 ppm, did not have a significant effect and was not considered to be suitable for use in ovitraps. We concluded that under laboratory conditions, the 10 ppm copper solution adequately killed mosquito larvae in a short period of time and would be useful for ovitraps.

Adult females did not show any effort to avoid the copper solution over the control. This will be a valuable point when we later use this solution in the field. The female mosquitoes of An. stephensi, Ae. albopictus, and An. sinensis randomly laid their eggs in the containers, which suggested that they could not detect or avoid the 10 ppm concentration of copper in the water. Therefore, it may be possible to get wild mosquitoes in the field to lay eggs in ovitraps containing 10 ppm of copper. The use of attractants that promote oviposition, as reported previously (Njiru et al., 2006), may further increase the possibility of success.

Confirmation in the field is clearly necessary before this method is applied. Researchers in Australia examining the use of ovitraps to kill mosquito larvae in

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**Table 1.** The number of eggs laid in cups containing 10 ppm copper or copper free (0 ppm).

|                | Anopheles stephensi | Aedes albopictus | Anopheles sinensis |
|----------------|---------------------|------------------|--------------------|
|                |                     |                  |                    |
|                | 0 ppm               | 10 ppm           | 0 ppm              | 10 ppm                |
| No. of eggs    | %1) 29.1            | 20.9             | 29.4               | 20.6                  |
|                | No. of eggs         |                  | No. of eggs        |                       |
| Exp 1          | cup 1               | 515              | 492                | 30.6                  |
|                | cup 2               | 478              | 492                | 28.5                  |
| Exp 2          | cup 1               | 19               | 291                | 31.2                  |
|                | cup 2               | 270              | 291                | 28.9                  |
| Exp 3          | cup 1               | 488              | 371                | 34.3                  |
|                | cup 2               | 497              | 371                | 34.9                  |
| Exp 4          | cup 1               | 286              | 159                | 42.1                  |
|                | cup 2               | 214              | 159                | 31.5                  |
| Total          |                     | 2767             | 2796               | 1948                  |
| Average        |                     | 29.1             | 20.9               | 29.4                  |

1), 2) and 3): In each species, no differences were observed between 0 ppm group and 10 ppm group by Mann–Whitney U-test.
homes reported high public acceptability for Standard Lethal Ovitraps and Biodegradable Lethal Ovitraps (Ritchie et al., 2009). Field tests showed that the ovitraps effectively targeted gravid *Ae. aegypti*. Another study demonstrated a significant impact on mosquito populations and suggested that ovitraps should be utilized in the field for dengue control (Rapley et al., 2009). Applications in the field will require a concerted effort to increase the effectiveness of these traps, such as considering the number of abiotic factors for their efficacy (Williams et al., 2006). The consideration of ovitrap colors and patterns to increase efficacy will also produce better results (Hoel et al., 2011).

We believe that a low concentration of copper can be used as an alternative for IVM along with the other chemical–insecticides or bio-insecticides for which problems have been reported with toxicities and resistances (Tetreau et al., 2012). Another safe and low cost alternative is needed in developing and undeveloped countries, as reported previously in Bangladesh (Rahman, 2012). This has encouraged us to suggest a 10 ppm copper solution as an alternative to be utilized in ovitraps. Although several environmental issues are also associated with copper, the careful utilization and strict control of low concentrations and limited usage in containers (ovitraps) will avoid contamination in the environment.

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Conflict of Interest

The authors have no conflict of interest to declare.

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